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THE UNIVERSITY OF ALBERTA

CHEMICAL MODIFICATION STUDIES OF PHOSPHORYLASE

IN THE CRYSTALLINE AND SOLUBLE STATES

by



EUNICE C.Y. LI CHAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1977

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHEMICAL MODIFICATION STUDIES OF PHOSPHORYLASE IN THE CRYSTALLINE AND SOLUBLE STATES submitted by EUNICE C.Y. LI CHAN in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Glycogen phosphorylase from rabbit muscle has been studied with respect to its reactions with 2,3-butanedione, potassium ferrate, water-soluble carbodiimide in the presence of a nucleophile, and diethyl pyrocarbonate. Techniques of solution protein chemistry were supplemented with the powerful tool of x-ray crystallography to investigate the sites and effects of these chemical modifications.

A functional arginyl residue at the anion-binding site was identified by selective modification with butanedione. The course of inactivation generally followed pseudo-first order kinetics and was first-order with respect to butanedione concentration. The rate of inactivation was slightly greater for phosphorylase a than b. Ultra-centrifugal studies, amino acid analyses and the reversibility of inactivation on dilution all confirmed selective modification of several arginyl residues. Protection from inactivation was provided by glucose-1-P, AMP and glucose-1-P, and UDP-glucose, but not by glycogen. The rate of inactivation of phosphorylase b was also retarded by AMP. The K_m of glucose-1-P for phosphorylase a remained constant over the course of inactivation, while the K_m values of glucose-1-P and AMP for phosphorylase b increased. Similar trends were observed in cross-linked tetragonal microcrystals of phosphorylase a. X-ray crystallography at 6 Å resolution showed a large new peak of electron density at the end of a long side chain in the anion-binding site, which had been previously shown in native crystals to bind glucose-1-P, arsenate, UDP-glucose and AMP. Ligand binding studies of butanedione-treated crystals showed a lower occupancy of AMP at the anion-binding site; UDP-glucose failed to bind at that site but was still found at the glucose-binding locus.

The rapid inactivation of phosphorylase by ferrate was accompanied by the destruction of 2 to 4 tyrosyl residues and the formation of 2 cysteic acid residues. The K_m values of glucose-1-P for both phosphorylases a and b increased over the course of inactivation. An increase in the K_m value of AMP and a decrease in the homotropic cooperativity of AMP binding were also observed in partially inactivated phosphorylase b. Significant protection against inactivation of phosphorylase a was provided by AMP and glucose-1-P, AMP and glucose, or AMP, glucose-1-P and glucose. Qualitatively similar results were obtained in the microcrystals. No distinct features appeared in the difference Fourier maps calculated for ferrate-treated versus native crystals. However, ligand binding studies indicated the predominant location of glucose-1-P at the glucose-binding site and poor binding of both AMP and glucose-1-P at the anion-binding site of treated crystals.

In general, the reaction of microcrystals of phosphorylase a with carbodiimide paralleled that previously studied in solution. Complex kinetics of inactivation suggest sequential modification of 2 groups of differing reactivity. The inactivation rate was increased by AMP, alone or with UDP-glucose. It was decreased by UDP-glucose alone or by glucose. X-ray crystallography depicted a multiplicity of sites of reaction.

Diethyl pyrocarbonate inactivated both phosphorylases a and b. Several substrates and inhibitors failed to show any significant effect on inactivation. Although the selective reaction of histidyl residues was accomplished, as supported by the pH dependence of inactivation, reversibility by hydroxylamine treatment, and ultraviolet spectral characteristics, a great number of histidyl residues were modified and it was not possible to distinguish the essential ones, if any.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
2'-AMP	adenosine-2'-phosphoric acid
3'-AMP	adenosine-3'-phosphoric acid
AMP or 5'-AMP	adenosine-5'-phosphoric acid
ATP	adenosine-5'-triphosphate
BES	<u>N</u> , <u>N</u> -bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid
DEPC	diethyl pyrocarbonate
DHT	5-diazo-1-H-tetrazole
DTNB	5, 5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
G1P	glucose-1-P or D-glucose-1-phosphate
G6P	glucose-6-P or D-glucose-6-phosphate
IMP	inosine-5'-phosphoric acid
MgOAc	magnesium acetate
NAD	nicotinamide adenine dinucleotide
RNA	ribonucleic acid
UDPG	UDP-glucose or uridine diphosphate glucose
ϵ	molar absorptivity

Chapter I

Introduction

Glycogen phosphorylase (α -1,4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1.) plays an important role in carbohydrate metabolism and is subject to control by energy metabolism, hormones and nervous stimulation. A number of small ligands can affect the enzymic activity, the principal allosteric activator being AMP, which enables phosphorylase b to exhibit 80% of full activity. Glucose, glucose-6-P and ATP can each antagonize the activation by AMP. In addition to allosteric control, enzymic activity is controlled by the interconversion between inactive phosphorylase b and active phosphorylase a by phosphorylation and dephosphorylation of ser-14 which is dependent on a cascade of interconverting reactions. This covalent modification of a single residue is sufficient to bring about alterations in protein structure that permit catalytic activity in the absence of AMP, escape from the allosteric energy controls by AMP and ATP, and an increase in the tendency for tetramer formation.

Although intensive investigations have been carried out on the catalytic and allosteric phenomena demonstrated by phosphorylases a and b, as is documented by a recent comprehensive review (1), there is little definitive information on the chemical basis for these phenomena. The study of the reaction of sulfhydryl groups with various thiol reagents indicated that the nine sulfhydryl groups per monomer may be categorized into three classes - two nonessential fast-

reacting groups, two essential slow-reacting groups, and five nonreactive groups (2-5). Since the modification of the sulfhydryl groups producing inactivation was highly sensitive to structural alterations such as those induced by AMP or ATP and since the subunit structure of the enzyme was affected by thiol modification, one is inclined to suggest that although sulfhydryl groups are essential, they play a role in structural integrity and conformation rather than in the catalytic mechanism.

The carbamylation of ϵ -amino groups of lysyl residues (6) produced inactivation, but again since inactivation was accompanied by protein dissociation, no conclusive statements could be made on the involvement of these ϵ -amino groups in catalysis. Reaction with glutaraldehyde and aliphatic aldehydes modified amino groups without greatly affecting structure or activity of the enzyme (7).

Apart from the studies on sulfhydryl and ϵ -amino groups, little work has surfaced to date regarding the importance of functional groups in phosphorylase. Several potential candidates involved in catalysis and substrate binding have been suggested. Detailed studies of pH and temperature effects on the kinetics of phosphorylase (8) suggest a group with pK near 6 responsible for the acid limb and a group with pK near 7.1 responsible for the alkaline limb of maximal velocity profiles. The phosphoryl moiety of enzyme-bound pyridoxal-5'-phosphate is proposed to be a candidate for the pK 6 group, but a carboxyl group in a hydrophobic environment may also fulfill this role. The imidazolyl group of a histidyl residue has been suggested to be a likely candidate for the pK 7.1 group. Some preliminary work

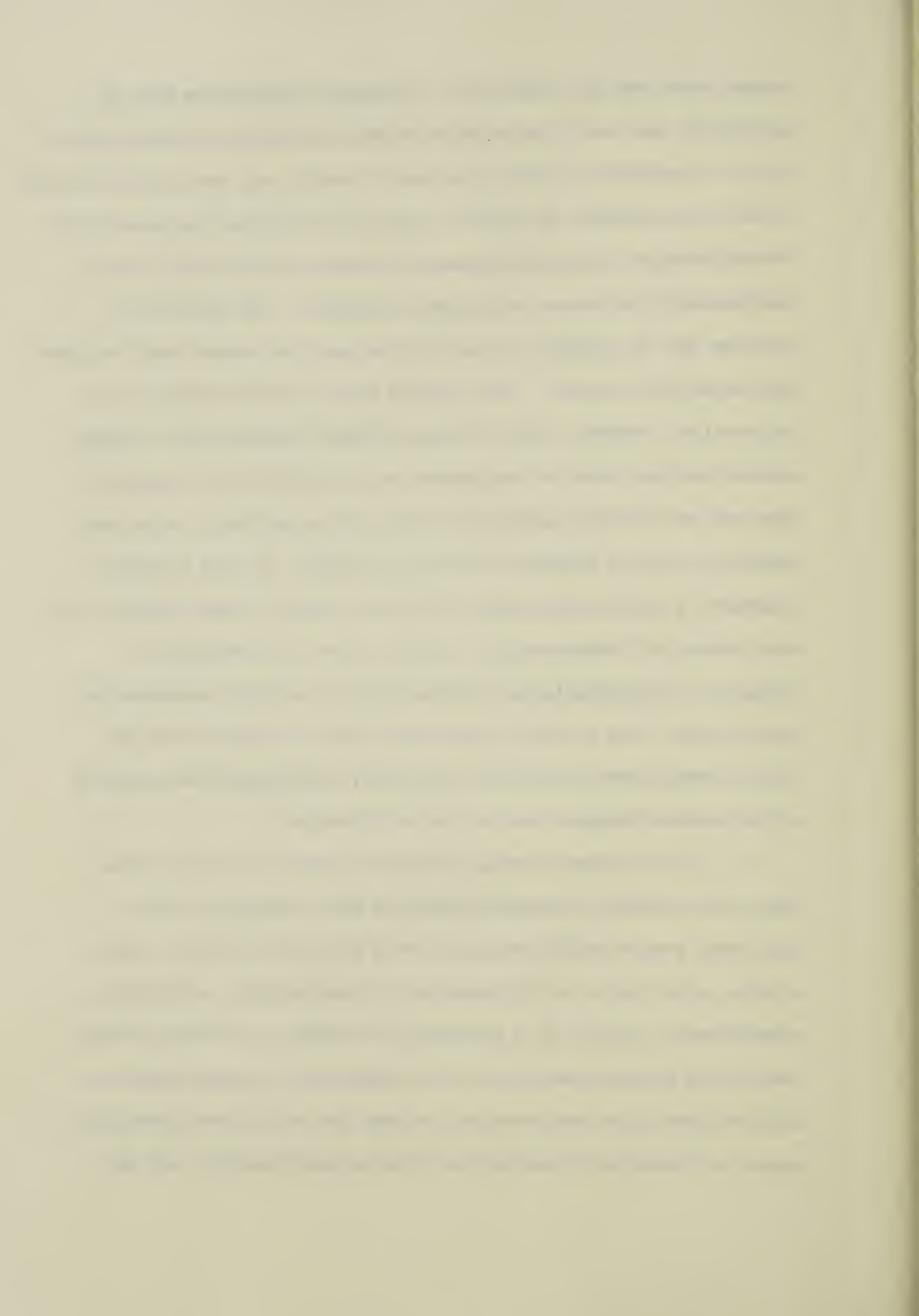
has confirmed the importance of histidyl residues in phosphorylase (9-11), but clear interpretation of these studies has been hindered by non-specificity and side reactions.

Studies of the AMP binding site of phosphorylase b have been carried out using covalent attachment of AMP analogues such as 6-(purine 5'-ribonucleotide)-5-(2-nitrobenzoic acid)thioether (12) and 8-(m-(m-fluorosulfonylbenzamido)benzylthio)adenine (13, 14), and have implicated the involvement of tyrosyl residues at or near the AMP binding site. A more recent example of using active site-directed substrate analogues is the work of Lee and Benisek (15). These authors employed potassium ferrate, a strong oxidizing agent which is also a phosphate analogue; tyrosine was suggested as the target of the inactivating chemical reaction in phosphorylase b, and the AMP site as the location of inactivation.

Clearly, much more work is required to gain further insights into the chemical basis for the various properties of this complex enzyme system. The characterization of the functional groups at the binding and catalytic sites of an enzyme is essential for an understanding of the enzymic activity at the molecular level. Chemical modification with reagents which selectively act on a limited number of reactive groups without causing irreversible denaturation is one approach by which this characterization may be studied. Changes in the activity or other properties of the enzyme on treatment with the reagent suggest a functional role of the modified residue(s). However, it is generally not immediately clear whether the effects result directly from modification at the active site or indirectly from conformational

changes away from the active site. Problems arising from lack of specificity are particularly relevant when a protein of large molecular weight is concerned. Even in the case in which only one particular type of amino acid residue is modified, there still remains the possibility of modification of many non-essential residues, which could lead to inactivation from loss of structural integrity. One approach to overcome this is through the use of site-specific rather than functional group-specific reagents. This usually ensures modification at the active site. However, rabbit muscle glycogen phosphorylase contains several distinct sites of importance in its activity and regulation - high and low affinity nucleotide binding sites, activator sites and inhibitor sites in addition to the active site. In such a complex oligomeric protein with several different types of ligand binding loci, even the use of "site-specific" reagents does not eliminate the ambiguity of interpreting an observed loss of activity accompanying modification. The problem is magnified when reversibility of the inactivating reaction precludes isolation, purification and analysis of the protein fragment bearing the modification.

In the present work, the study of some of the functional amino acid residues in phosphorylase has been carried out using functional group-specific reagents, such as 2,3-butanedione, water-soluble carbodiimide in the presence of a nucleophile, and diethyl pyrocarbonate, as well as a site-specific reagent, potassium ferrate. Some of the problems associated with conventional protein chemistry outlined above have been resolved through the use of x-ray crystallography to "directly" visualize the sites of modification, and the



effects of the modification on ligand binding. The x-ray diffraction studies on phosphorylase a have now produced a structure at 3.0 Å^o resolution, and have provided a description of the binding sites for substrates, competitive inhibitors and allosteric effectors (16). Since the activity of phosphorylase in the microcrystalline form has also now been characterized (17), parallel studies of chemical modification in the microcrystals compared to the enzyme in solution may provide the necessary link to combine the methodology of protein chemistry with that of x-ray crystallography to examine the functional groups in phosphorylase.

Chapter II

Methods and Materials

1. General Protein Preparation and Characterization

Rabbit muscle phosphorylase b was prepared by the method of Fischer and Krebs (18) and recrystallized at least three times; the enzyme was purified on Sephadex G-25 equilibrated with the appropriate buffer prior to use. Phosphorylase a was prepared from phosphorylase b with phosphorylase b kinase according to Krebs et al (19). Protein concentration was determined from the absorbance at 280 nm, using the value of 13.2 for $E_{1\text{ cm}}^{1\%}$ (20). Enzyme activity was measured in the direction of glycogen synthesis under zero-order kinetic conditions, unless otherwise specified. Typically, the assays contained 50 $\mu\text{g/ml}$ enzyme, 75 mM glucose-1-P and 1% glycogen (21); 1 mM AMP was also present for assays with phosphorylase b. Preparation and assay of tetragonal glutaraldehyde cross-linked microcrystals of phosphorylase a were carried out essentially as described by Kasvinsky and Madsen (17).

Amino acid analyses were performed on a Spinco automatic amino acid analyzer after hydrolysis of samples in vacuo in 6N HCl containing 0.2 - 0.5% phenol at 110° for 24 hours. Sedimentation velocity runs were performed on a Spinco model E ultracentrifuge, and sedimentation constants were calculated from microcomparator measurements of the Schlieren diagram.

The collection of diffraction data for difference Fourier maps to 6 Å resolution or to 4.5 Å resolution for crystals of modified

phosphorylase a were carried out as described by Fletterick et al (22).

2. Reaction with Butanedione

2,3-Butanedione (diacetyl) was purchased from Aldrich; a 2% solution in sodium borate (pH 7.0) buffer is stable for several months if frozen. Arginine modification with butanedione was carried out at 30° in 50 mM borate-1 mM EDTA-5 mM DTT buffer adjusted to pH 7.5. Preliminary studies indicated that inactivation was negligible when the reaction was carried out at pH 6.8. The concentrations of the monomeric butanedione used were generally lower than those used by Riordan (23), who introduced the use of this reagent in borate buffer as a selective reagent for arginine modification. Unless otherwise specified, 5 mM and 10 mM butanedione were routinely used for reaction with 1 mg/ml phosphorylases a and b in the solution form, respectively; 10 mM butanedione was used for reaction with 0.5 mg/ml phosphorylase a in the cross-linked microcrystalline form. Aliquots of the reaction mixture were diluted to 10- to 40-fold with assay buffer (pH 6.8) to stop the reaction prior to assay of activity. Crystals of phosphorylase a were reacted with a 30-fold molar excess of butanedione in 50 mM borate-10 mM BES-1 mM EDTA-5 mM DTT pH 7.5 buffer for four hours at 30°. The reaction was then terminated by repeated changes with fresh borate buffer. Ligand binding studies were carried out by further soaking the modified crystals with added ligand in the above buffer. Analogous experiments were carried out with phosphorylase b in solution and phenylglyoxal, purchased from Aldrich.

The effects of ligands on enzyme inactivation by butanedione

were carried out by addition of the ligand(s) to the enzyme prior to reaction with butanedione. Glucose-1-P and enzyme solutions were treated with α -amylase to remove traces of long-chain polysaccharides and to prevent catalysis during studies of the protecting effects of glucose-1-P or AMP and glucose-1-P.

Samples for amino acid analyses were prepared by addition of aliquots of reaction mixtures to equal volumes of 6N HCl containing 0.5% phenol for a 24 hour hydrolysis in vacuo at 110° prior to amino acid analysis.

Aliquots of the reaction mixture containing 3 mg/ml phosphorylase and 20 mM butanedione were prepared for sedimentation velocity runs by stopping the reaction with the addition of arginine-HCl (pH 6.8) in a 2-fold molar excess over butanedione. Preliminary experiments showed that this method effectively stopped the reaction. Controls were treated similarly except for the exclusion of butanedione.

3. Reaction with Potassium Ferrate

Potassium ferrate was prepared as described by Thompson et al (24). The purity of the preparation was determined to be about 90% by the chromite method (25). Since rapid oxidation of water by ferrate occurs at pH below 10, ferrate is unstable in neutral aqueous solutions. Stock solutions were thus prepared fresh, prior to each experiment, in 0.1 mM NaOH under nitrogen at 4° and centrifuged to remove any ferric hydroxide precipitate which had formed.

Stock solutions of three times recrystallized enzyme freed of AMP by passage through Sephadex G-25 were dialyzed against 5 mM

sodium cacodylate pH 7 buffer under nitrogen at 4° to remove reducing reagents such as 2-mercaptoethanol or dithiothreitol which could react with ferrate. These thiol-free stock solutions were stored under nitrogen and used within a week of preparation. Cross-linked microcrystals of phosphorylase a were freed of reducing reagents by exhaustive washing, achieved by alternately spinning down the microcrystals at very low speeds in a clinical centrifuge and resuspending in fresh buffer.

The reaction was initiated by the addition to the enzyme of a small aliquot of the freshly prepared ferrate solutions in 0.1 mM NaOH. The reaction was stopped at the desired time by addition of assay buffer containing mercaptoethanol to final concentrations of 10 mM.

Ferrate was added to crystals of phosphorylase a in 5 mM sodium cacodylate buffer until a mole ferrate per mole a monomer ratio of 30 was achieved. After a two hour reaction, the crystals were washed with fresh cacodylate buffer, and for ligand binding studies, further soaked in buffer containing the desired ligand.

Prior to acid hydrolysis of ferrate-treated phosphorylase a in preparation for amino acid analysis, the reaction mixtures were extensively dialyzed against 0.1 M potassium phosphate-1 mM EDTA-25 mM 2-mercaptoethanol buffer at pH 7.0, and then exhaustively dialyzed against glass-distilled water.

4. Reaction with Carbodiimide

Glycine ethyl ester was purchased from Aldrich Chemical

Company, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate was purchased from Sigma. The carbodiimide and nucleophile solutions were freshly prepared and adjusted to pH 6.7 for each experiment. When both carbodiimide and nucleophile were to be included in the reaction, the carbodiimide was first dissolved in the pH 6.7 glycine ethyl ester solution, and then the pH of this solution was re-adjusted to 6.7 prior to addition to the enzyme.

The reactions were carried out on the solution form of the enzyme in 10 mM glycerophosphate-1 mM EDTA-2.5 mM DTT pH 6.7 buffer, and on the microcrystals or crystals of the enzyme in 5 mM BES-0.5 mM EDTA-2.5 mM DTT pH 6.7 buffer.

5. Reaction with Diethyl Pyrocarbonate

Diethyl pyrocarbonate (DEPC) was purchased from Sigma and stored at 4° in the presence of a dessicant to prevent decomposition into ethanol and carbon dioxide in the presence of moisture. The purity of different batches of DEPC was checked using $^{\alpha}$ N-acetylhistidine, as described by Holbrook and Ingram (26), except that glycerophosphate buffer was used instead of phosphate buffer. All experiments on phosphorylase b were carried out with the same batch of DEPC. No detectable change in reagent purity occurred during the four weeks necessary to complete the set of experiments. A second batch of DEPC was used for the experiments performed on phosphorylase a. This batch had already undergone considerable decomposition, as evidenced by the accumulation of gas which was rapidly released on opening the bottle. The preliminary work reported here on phosphorylase a

was completed within two days of opening the bottle of reagent, during which time the reagent purity was assayed to have decreased to about 70%.

Chapter III

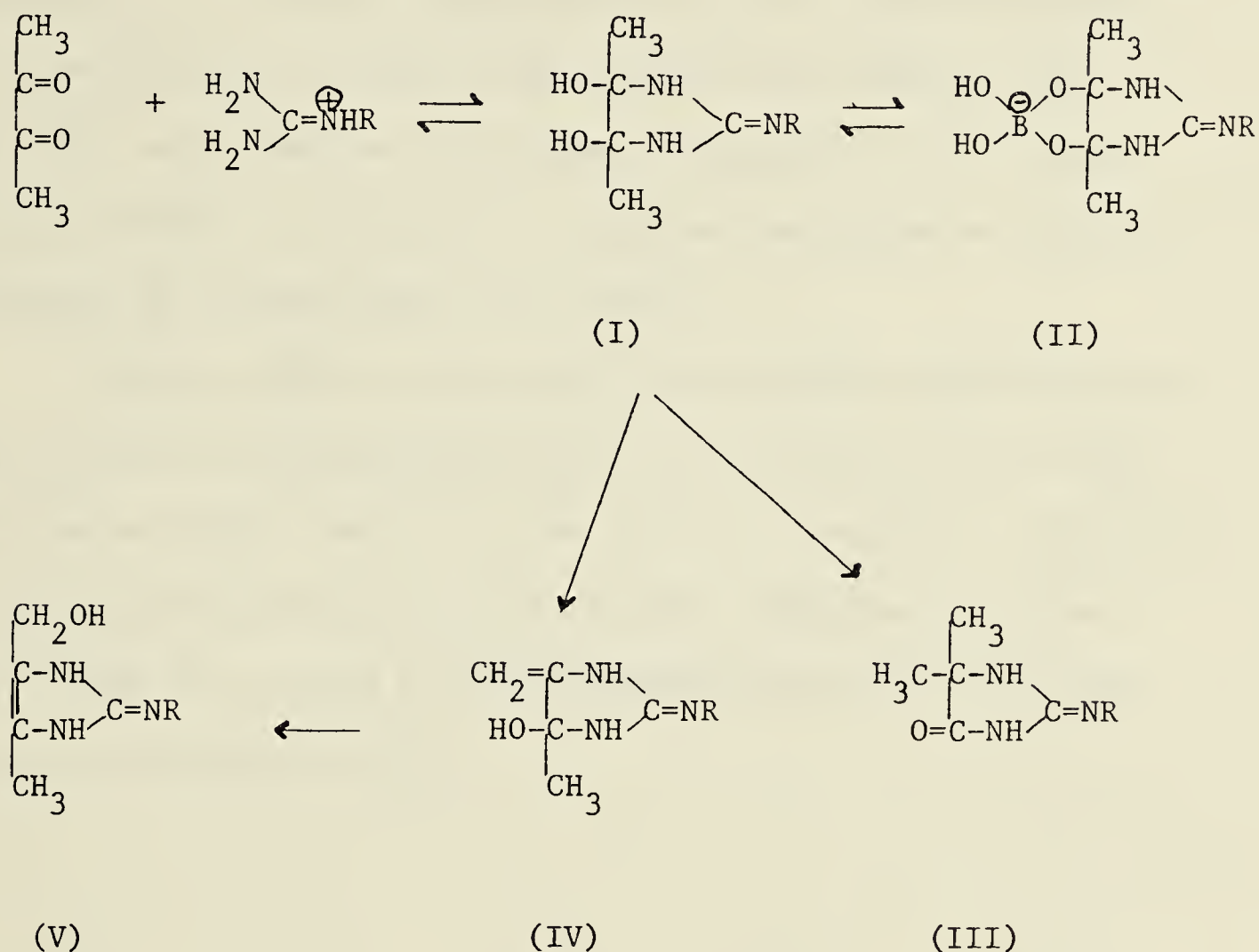
Modification of Arginyl Residues with Butanedione

A. Introduction

Arginyl residues can, through their positively charged guanidinium group, act as recognition or binding sites for negatively charged substrates or cofactors in enzyme active sites. Recent work on several enzymes such as alkaline phosphatase (27), aldolase (28), fructose-1, 6-bisphosphatase (29, 30) and glutamine synthetase and carbamyl phosphate synthetase (31) have illustrated the importance of arginyl residues at the binding sites for substrates or cofactors containing a phosphate moiety. Riordan et al (32) showed that nine out of ten glycolytic enzymes studied contain arginyl residues at their active site. These authors also pointed out the characteristics of the guanidinium group which make it ideally suited for interaction with phosphorylated metabolites - its planar structure and ability to form multiple hydrogen bonds with the phosphate moiety. Resonance stabilization of the guanidinium group, reflected by its high pK, make it an unlikely general acid catalyst for hydrolysis of phosphorylated intermediates, thus ensuring maximum utilization of substrate phosphate for the synthesis of ATP for the overall efficiency of glycolysis. These studies all suggest the possibility of arginyl residues at the ligand binding sites of phosphorylase.

A number of α -dicarbonyl compounds have recently been found to be suitable reagents for the modification of arginyl residues in

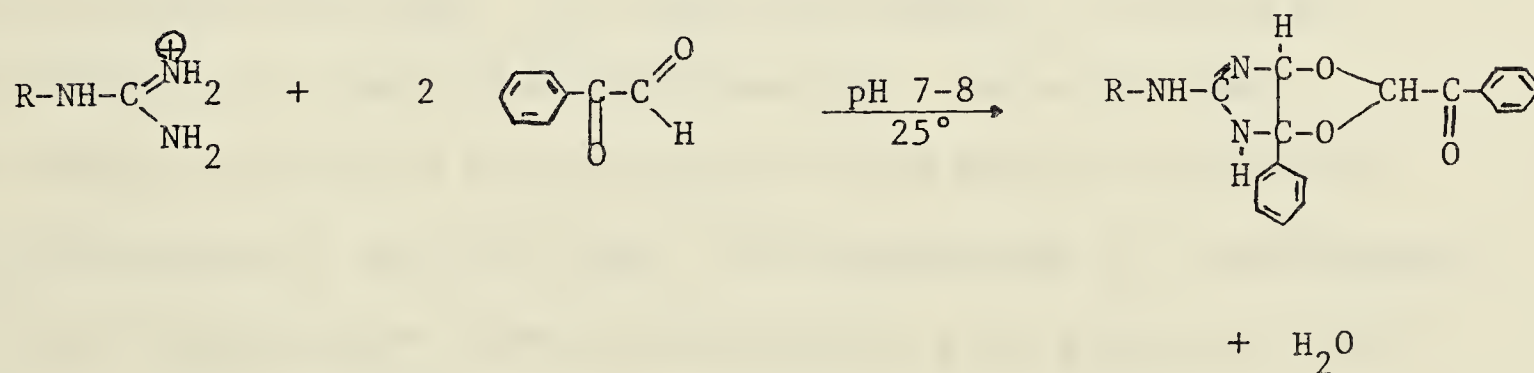
proteins. Among these is the reagent 2,3-butanedione, which in its monomeric form reacts specifically and under relatively mild conditions (23). The following scheme, adapted from Riordan (23), illustrates the possible products of reaction:



The reaction of butanedione with the guanidinium group to form the imidazoline derivative (I) is freely reversible. The reaction rate is enhanced by product stabilization by a reversible complex formation with borate ion (II). In the absence of excess butanedione and borate for stabilization, the reversibility of these steps allows the slow dissociation of (I) or (II) to regenerate free arginine. However,

prolonged treatment with butanedione may lead to an irreversible rearrangement to product (III) which is no longer dependent on borate. Acidification of the reaction mixture will lead to the irreversible formation of a methylene intermediate (IV) and the hydroxymethyl-imidazoline (V). Thus the acid hydrolysis of the reaction mixture which is prerequisite to amino acid analysis will probably give product (V), which does not break down to regenerate free arginine. It should therefore be possible to determine the degree of arginine modification by routine amino acid analysis.

Another reagent found useful for selective modification of arginyl residues is phenylglyoxal (33). This reaction also occurs under relatively mild conditions, and is also reversible. Reaction of a second phenylglyoxal molecule provides stabilization of the product formed on reaction of the guanidinium group with the first molecule of phenylglyoxal (34).



B. Results

1. Kinetics of Inactivation

Preliminary studies with phosphorylase b in solution showed that at pH 7.5, a complete loss of activity resulted within twenty minutes of addition of 2,3-butanedione in a 50-fold molar excess over arginyl residues. In subsequent work, lower reagent concentrations were used to encourage greater specificity of the reaction. Semilogarithmic plots for the course of inactivation of phosphorylases a and b at various butanedione concentrations are shown in Figures 1A and 1B. The rate and kinetics of inactivation may be affected by the rate of stabilization of the butanedione-guanidinium complex by borate. Deviation from pseudo-first order kinetics may occur when the rate of formation of the butanedione-guanidinium complex is limited by the rate of its stabilization by borate. Figure 2 shows the effect of butanedione concentration on the pseudo-first order rate constant of inactivation, k , obtained from the initial linear positions of the semilogarithmic plots in Figures 1A and 1B. The linear relationship is expected for the behaviour of a simple bimolecular reaction. The second order rate constants calculated from the slopes of the plots are $14 \text{ M}^{-1} \text{ min}^{-1}$ for phosphorylase a, and $6.3 \text{ M}^{-1} \text{ min}^{-1}$ for phosphorylase b. The difference in rates of inactivation between phosphorylases a and b may be a result of conformational changes from phosphorylation of the ser-14 residue. The conformation of the binding site in phosphorylase a which provides greater affinity for AMP or glucose-1-P, compared to phosphorylase b, may also favor reaction with butanedione.

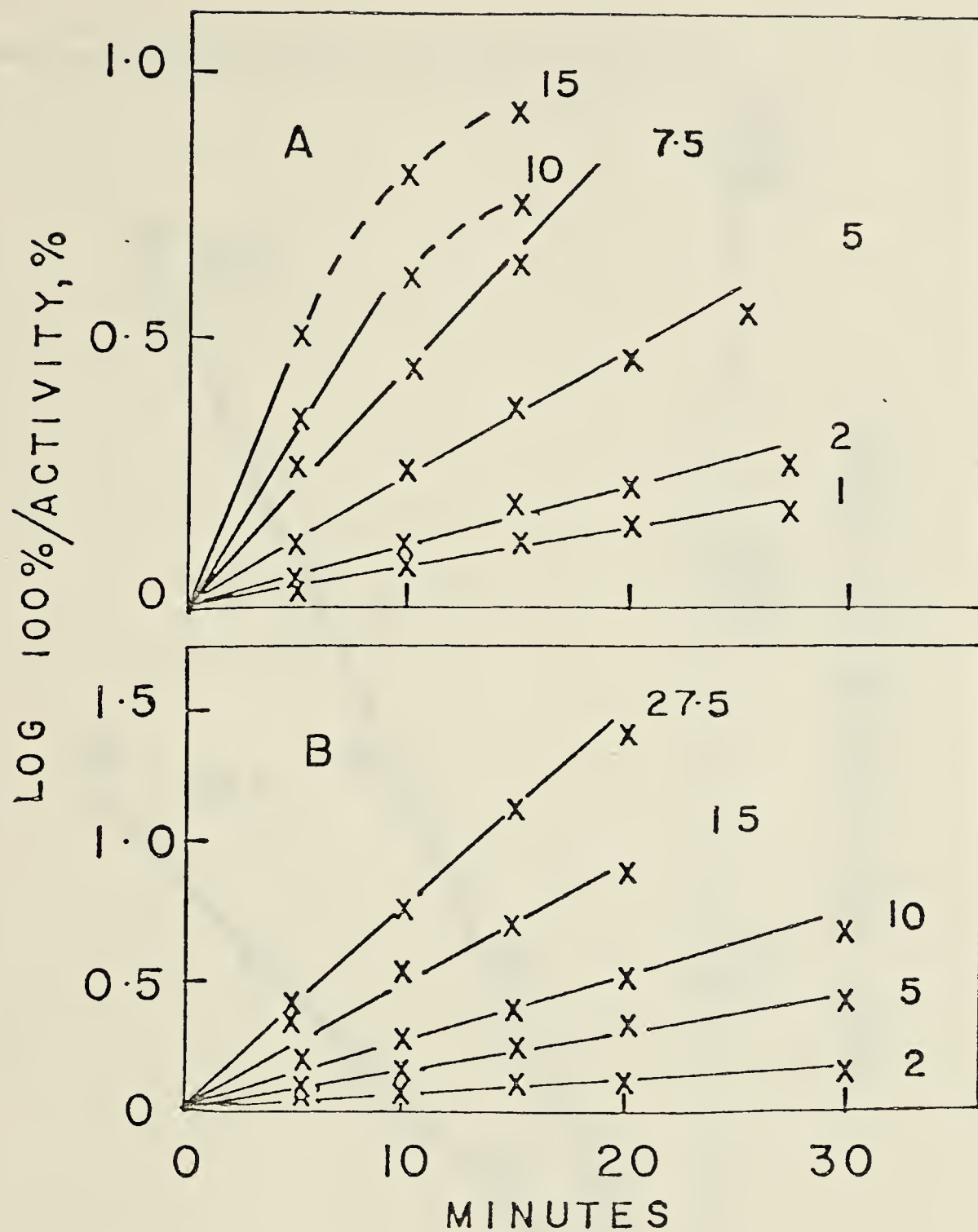


Figure 1.

Semilogarithmic plots of the course of inactivation of (A) phosphorylase a and (B) phosphorylase b on reaction with the indicated concentrations of butanedione (mM) in 50 mM borate-1 mM EDTA-5 mM DTT pH 7.5 buffer. Enzyme concentration was held constant at 1 mg/ml.

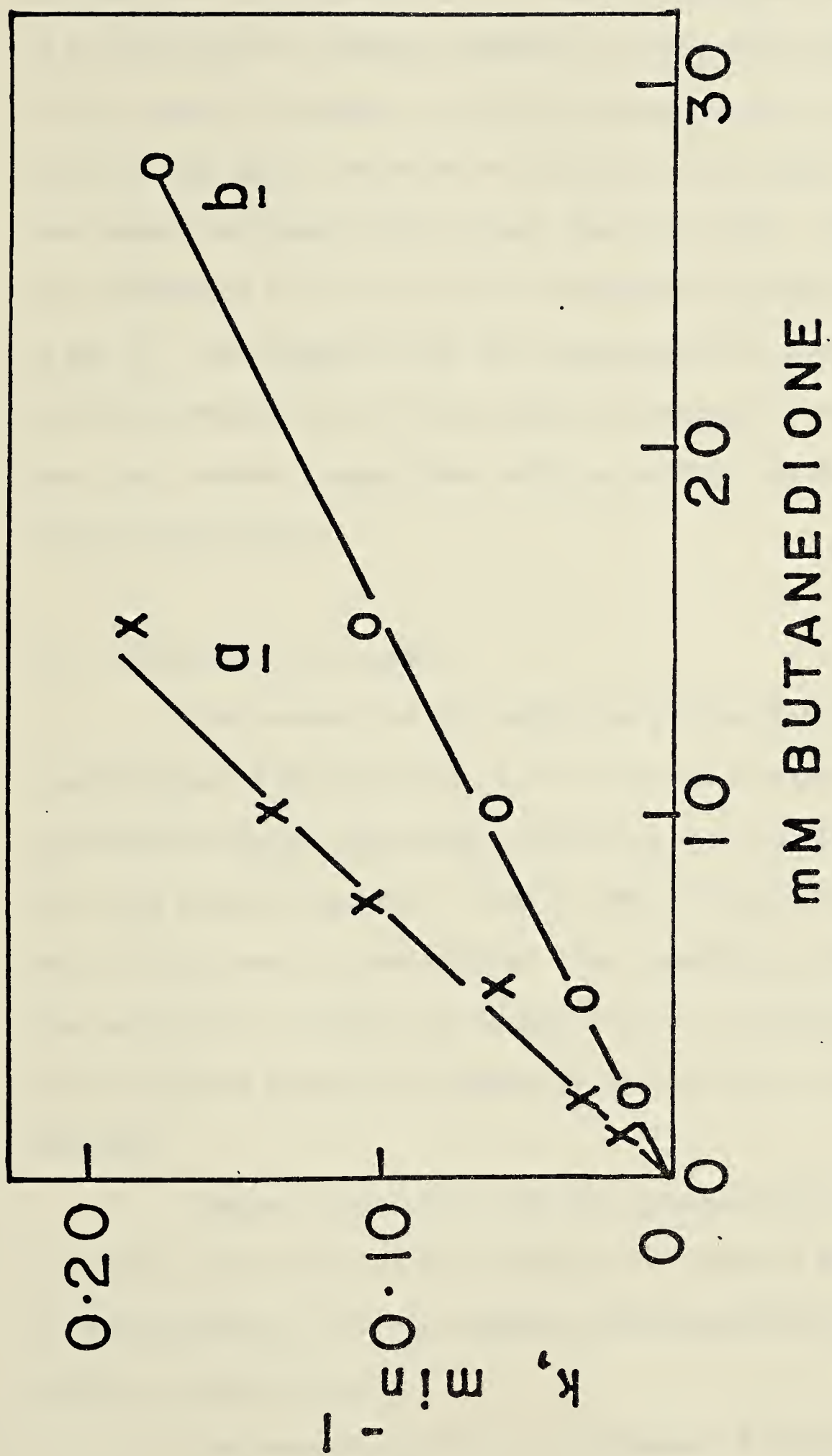


Figure 2.

Effects of butanedione concentration on the pseudo-first order rate constants of inactivation (k) of phosphorolases a and b.

Plots of $\log (1/\text{half-time of inactivation})$ against \log (butanedione concentration) were used to determine the minimum number \underline{n} of molecules of reagents required to react with each active unit of the enzyme to produce an inactive enzyme-reagent complex. This type of plot gives the order of reaction with respect to the reagent and should be linear with a slope equal to \underline{n} (35). The value of \underline{n} was determined to be 0.9 for both butanedione-treated phosphorylases a and b. This suggests that the inactivation by butanedione is the result of modification of one arginyl residue per active site. It does not, however, prove that only one arginyl residue has reacted to cause inactivation.

2. Protection With Ligands

The presence of the substrate glucose-1-P decreased the inactivation of phosphorylase a by butanedione (Figure 3A). Even greater protection resulted when both glucose-1-P and the allosteric activator AMP were present together. However, AMP by itself caused slight enhancement of the rate of inactivation. The competitive inhibitor UDP-glucose was also able to protect the enzyme and at a concentration of 5 mM was able to afford protection comparable to that with 35 mM glucose-1-P and AMP.

Similar trends were seen for phosphorylase b (Figure 3B). In addition, AMP as well as its analogue IMP afforded some protection. At a concentration of 1%, the substrate glycogen did not have any appreciable effect on inactivation.

The protecting effects of glucose-1-P and other ligands were

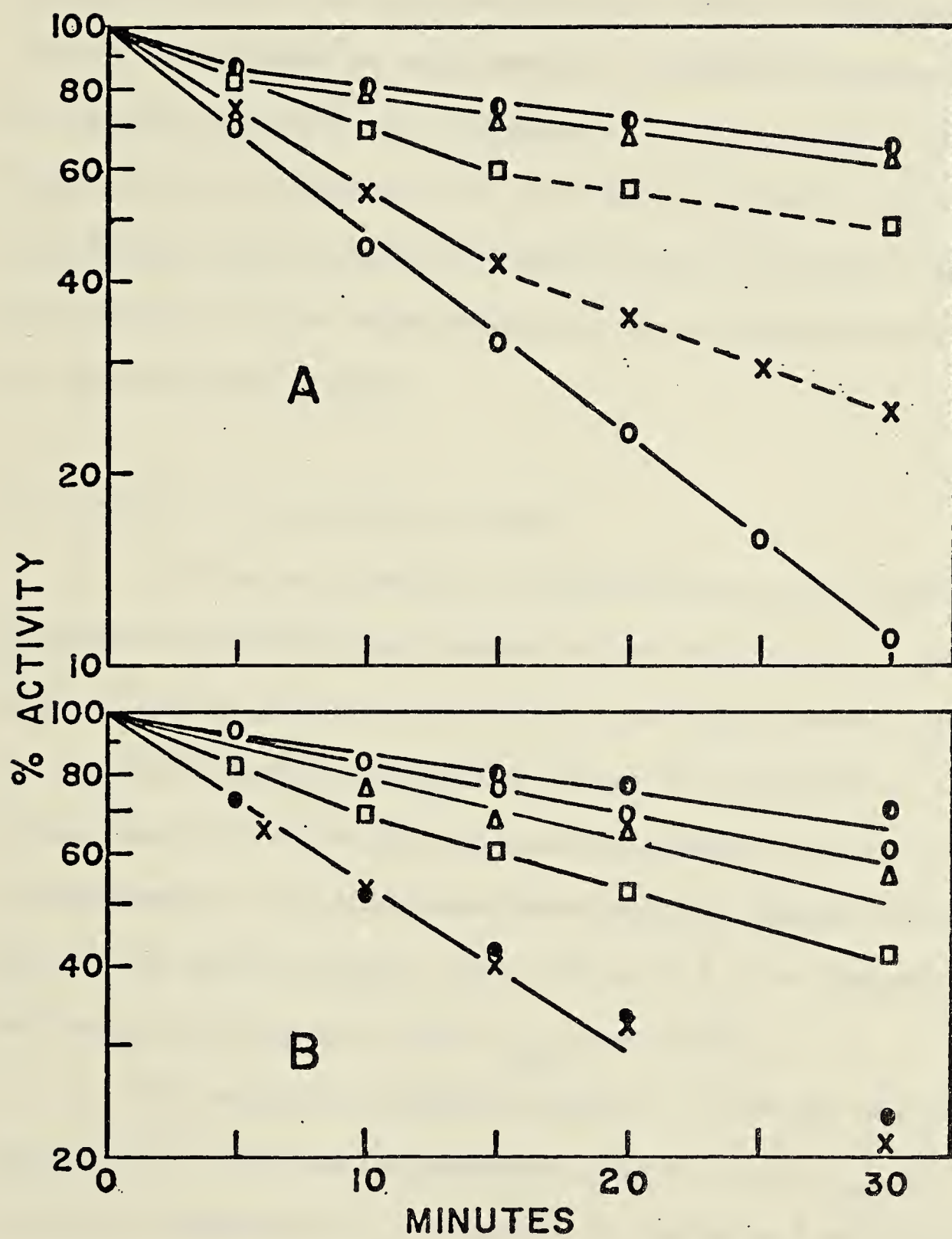


Figure 3

Effects of ligands on the course of inactivation by butanedione.

(A) Reaction mixtures contained 1.0 mg/ml phosphorylase a and 5 mM butanedione with the following ligands: X, none; 0, 1 mM AMP; □, 35 mM glucose-1-P; ●, 1 mM AMP and 35 mM glucose-1-P; Δ, 5 mM UDP-glucose.

(B) Reaction mixtures contained 1.0 mg/ml phosphorylase b and 10 mM butanedione with the following ligands: X, none; ●, 1% glycogen; □, 37.5 mM glucose-1-P; Δ, 2 mM IMP; 0, 1 mM AMP; ●, 1 mM AMP and 37.5 mM glucose-1-P.



not due to changes in ionic strength of the solutions, since the addition of 75 mM NaCl had a negligible effect on the inactivation process. Complete protection against inactivation was not observed although the rates of inactivation were reduced by the protecting ligands. This might be explained by a competition between ligand and reagent molecules for a common site on the enzyme. Since the reagent bonds covalently to an amino acid residue at the site, it is expected that eventually it would be able to displace the protecting ligand, the rate being influenced by the dissociation constant of the enzyme-ligand complex.

3. Activity of the Modified Enzyme

If the inactivation of phosphorylase were a result of specific blocking or conformational changes at the active site to produce a modified enzyme with partial activity, one might expect a change in the kinetic properties during the course of inactivation. Alternatively, if the reaction were an "all-or-none" phenomenon leading to two types of phosphorylase, totally inactivated modified enzyme and unmodified enzyme with native activity, then values of K_m for glucose-1-P should not change significantly, while V_{max} does change.

The results are shown in Table 1. With the concentration of glucose-1-P as the varying parameter, the value for V_{max} was reduced 5-fold for phosphorylase a (1 mg/ml) treated with 5 mM butanedione for 30 minutes; K_m did not change significantly. For phosphorylase b (0.5 mg/ml) a 10-fold decrease in V_{max} resulted on reaction with 10 mM butanedione for 30 minutes; K_m was increased 2.5-fold. The K_m

Table 1
Kinetic parameters for phosphorylases
partially inactivated by butanedione

Minutes of exposure to butanedione	Phosphorylase <u>a</u>		Phosphorylase <u>b</u>			
	K_m of GlP ^{b,c}	V_{max} with varied GlP ^c	K_m of GlP ^d	V_{max} with GlP ^d	K_m of AMP ^e	V_{max} with varied AMP ^e
0	2.8	69	2.7	45	3.6×10^{-2}	59
7 1/2	2.0	41	4.4	23	-	-
10	-	-	-	-	6.5×10^{-2}	22
15	1.9	26	5.6	12	-	-
30	1.9	16	6.7	4	-	-

NOTE: Units for K_m are millimolar and for V_{max} are $\mu\text{moles/min/mg}$ at 30°

^aButanedione concentrations were 5 mM for 1 mg/ml phosphorylase a
and 10 mM for 0.5 mg/ml phosphorylase b.

^bdetermined from Hill plots

^cdetermined at 1% glycogen

^ddetermined at 1% glycogen, 1 mM AMP

^edetermined at 1% glycogen, 24 mM glucose-1-P

for AMP was also increased for phosphorylase b, a 2-fold increase resulting after 10 minutes reaction. This result suggests that in phosphorylase b, the reagent may be affecting the binding site of AMP either directly by reacting there, or indirectly by steric hindrance or conformational changes. Since cooperative heterotropic interactions occur between AMP and glucose-1-P, the changes in K_m for glucose-1-P may reflect changes in this phenomenon rather than a deviation from an all-or-none reaction at the glucose-1-P site.

To further investigate the possibility of a partially active modified enzyme, the activity of butanedione-treated phosphorylase b in the presence of AMP was compared to that in the absence of AMP. Phosphorylase b from rabbit skeletal muscle exhibits a virtually absolute requirement for nucleotide activation for catalytic activity (1). This activation is generally provided during in vitro assays by the addition of AMP. However, it is conceivable that chemical modification at the AMP binding site may trigger some of the events which occur on binding of the activator, allowing activity in the modified enzyme. In this case, it is assumed that the site of chemical modification is not also the active site.

The results in Figure 4 show that the activity of phosphorylase b in the absence of AMP did indeed increase significantly with extended periods of butanedione treatment, accompanied by a decrease in the activity assayed in the presence of AMP. Thus, the "minus AMP activity" of phosphorylase b increased from 0.84 $\mu\text{moles/min/mg}$ in the native enzyme to 4.5 $\mu\text{moles/min/mg}$ in enzyme after 3 hours of butanedione treatment, while the "plus AMP activity" decreased from

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2111. 2112. 2113. 2114. 2115. 2116. 2117. 2118. 2119. 2120.

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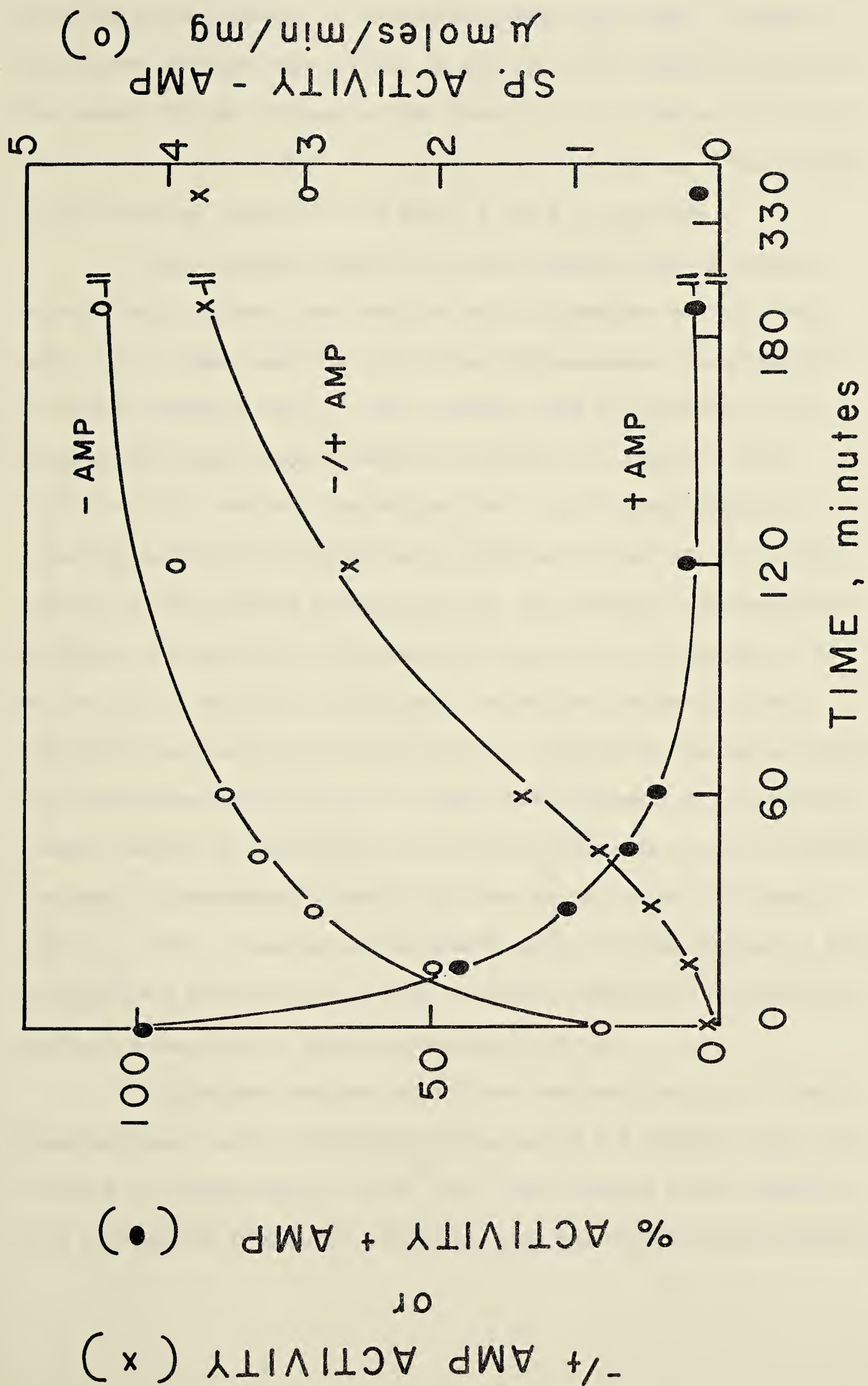
2181. 2182. 2183. 2184. 2185. 2186. 2187. 2188. 2189. 2190.

2191. 2192. 2193. 2194. 2195. 2196. 2197. 2198. 2199. 2200.

Figure 4.

Activity of butanedione-treated phosphorylase b as a function of time of reaction, assayed in the presence ("AMP") or absence ("AMP") of 1 mM AMP. Aliquots of the reaction mixture of 1 mg/ml enzyme and 10 mM butanedione in borate buffer at pH 7.5 were assayed for activity. The activities in the presence of AMP are expressed as a percentage of residual activity, on the basis of 100% in the native enzyme. The activities in the absence of AMP are expressed as the specific activity, the native enzyme possessing a specific activity of 0.84 μ moles/min/mg.

- % residual activity assayed in the presence of 1 mM AMP ("AMP")
- 0 specific activity in μ moles/min/mg, assayed in the absence of AMP ("AMP")
- X ratio of activity of butanedione-treated phosphorylase b assayed in the absence versus presence of 1 mM AMP ("AMP")



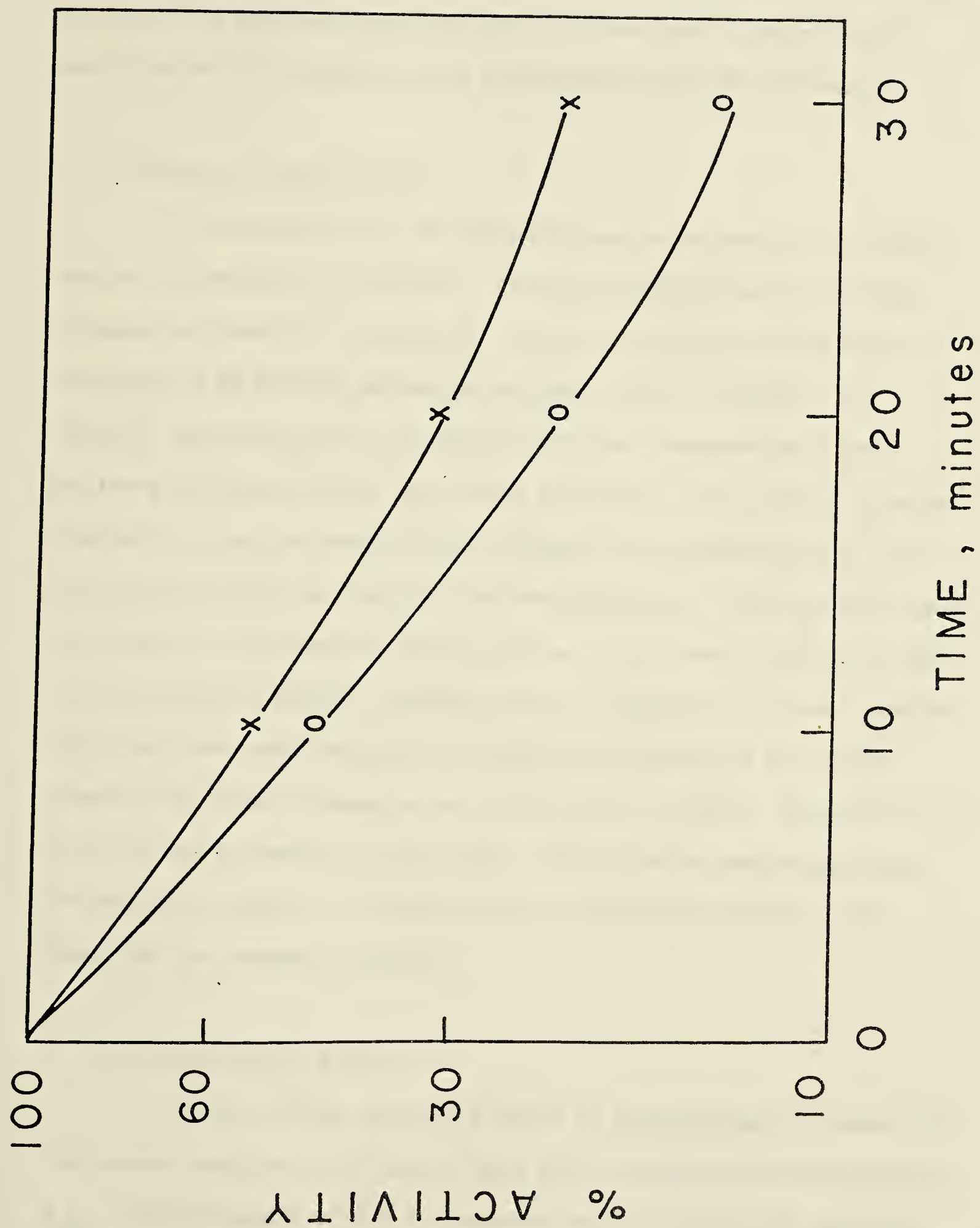
100% (84 μ moles/min/mg) to 5% over the same time period. Figure 4 also shows the same data plotted as the ratio of activity assayed in the absence versus presence of AMP (denoted as "-/+AMP activity") as a function of the time of the reaction with butanedione. The -/+AMP ratio increased from 1% to 90% after 3 hours of reaction.

These results clearly show that phosphorylase b remains catalytically active after reaction with butanedione and that this activity no longer exhibits the strong AMP dependence characteristic of native phosphorylase b. This suggests that the binding of the butanedione-borate complex mimics the effects of binding of the activator AMP. However, the butanedione-treated phosphorylase b catalytic activity is rather poor. This may be due either to other effects of the chemical modification on, for example, conformational stability and mobility or structural integrity of the enzyme, or it may be due to the lack of structural resemblance between the more efficient "natural" activator AMP and the chemically induced activator, the butanedione-borate complex. Since phenylglyoxal, an α -dicarbonyl reagent similar to butanedione in its specificity for arginyl residues, contains a six-membered aromatic ring and is thus more structurally similar to AMP, it was hoped that modification of phosphorylase b with phenylglyoxal would lead to a more efficient mimicking of nucleotide activation and thus to greater catalytic activity.

Preliminary studies showed that the inactivation of 1 mg/ml phosphorylase b with 10 mM phenylglyoxal at pH 7.8 (second order rate constant of inactivation = $7.6 \text{ M}^{-1} \text{ min}^{-1}$) was similar to the reaction with butanedione (Figure 5). However, phenylglyoxal-modified phosphory-

Figure 5.

Semilogarithmic plots of the time course of inactivation of 1 mg/ml phosphorylase b on reaction with 10 mM butanedione at pH 7.5 (X) and 10 mM phenylglyoxal at pH 7.8 (O).



lase b did not exhibit any increase in its minus AMP activity. This may be due to steric hindrance or improper orientation of the phenyl group at the activator binding site; alternatively, the sites of modification by butanedione and phenylglyoxal may be different.

4. Reversal of Inactivation

The inactivation of phosphorylase by butanedione in borate buffer is partially reversible. This was demonstrated by diluting aliquots of partially inactivated enzyme 20-fold into 20 mM glycerophosphate-2 mM EDTA-20 mM mercaptoethanol (pH 6.8) buffer. For example, phosphorylase a solutions which had possessed only 29% activity exhibited 57% of the native activity 22 hours after dilution (Table 2). Similar results were observed for phosphorylase b. The presence of borate is required for stabilization of the cis-diol formed on reaction of butanedione with arginine (23); thus, on dilution into glycerophosphate buffer, reversal would be expected to occur. Enzyme which had been modified in the presence of glucose-1-P and/or AMP showed only slight changes in activity after dilution. Restoration of activity was incomplete in all cases. This finding may suggest some irreversible changes at residues not at the active site but still essential for maximal activity.

5. Ultracentrifugal Studies

Sedimentation velocity studies on phosphorylase b showed that the enzyme remained in a dimeric form after treatment with butanedione; S_{obs} values remained at 8.5 for samples having 40% and 16% residual

Table 2. Reversibility of butanedione inactivation by dilution.

Phosphorylase solutions and glucose-1-P solutions were amylase-treated prior to reaction with butanedione. Reversibility of the inactivation process was determined by 20-fold dilutions of aliquots of partially inactivated phosphorylase solutions into 20 mM glycerophosphate-2 mM EDTA-20 mM mercaptoethanol (pH 6.8) buffer. The diluted enzyme was assayed immediately and after 22 hours at 22°C. Numbers in parentheses indicate the activity of the controls after 22 hours relative to their initial activity.

Table 2

Reversibility of butanedione inactivation by dilution

Conditions	Time of Reaction, minutes	Initial % Activity	% Activity after 22 hr, 22°
1 mg/ml phosphorylase <u>a</u> and 5 mM butanedione			
I. no other ligands	0	100	"100" (94)
	10	43	65
	20	29	57
II. with 35 mM G1P	0	100	"100" (93)
	10	69	69
	20	55	59
III. with 1 mM AMP and 35 mM G1P	0	100	"100" (96)
	10	81	77
	20	72	70
1 mg/ml phosphorylase <u>b</u> and 10 mM butanedione			
I. no other ligands	0	100	"100" (94)
	10	55	77
	20	28	51
II. with 1 mM AMP	0	100	"100" (96)
	10	80	85
	20	69	78
III. with 1 mM AMP and 37.5 mM G1P	0	100	"100" (96)
	10	83	91
	20	80	85

activity. The single peaks and S_{obs} values thus support the view that the decrease in activity on reaction with butanedione was not a consequence of changes in the monomer-dimer-tetramer equilibrium, but to other changes, perhaps at the catalytic or ligand-binding sites of the enzyme.

6. Amino Acid Analysis

Accurate detection of modification of a few arginyl residues is precluded in a protein of this size, but an indication of the general trends of the reaction can be obtained. Amino acid analyses of the modified enzyme indicated no significant loss of any amino acid residues other than arginine, and no anomalous peaks were detected. Assuming that the acidification of the reaction mixture can prevent regeneration of free arginine (23), the decrease in arginine content corresponds to the number of modified arginyl residues. Table 3 shows that relatively large numbers of arginyl residues were lost in modified enzyme exhibiting 25-50% activity. From 5 to 15% of the total number of arginyl residues present in the native enzyme were lost. However, this is not unexpected due to the large size of the protein and the number of accessible residues on the surface of the molecule. The number of arginyl residues lost was reduced when the reaction was carried out in the presence of glucose-1-P or of AMP, suggesting the possibility of protection of specific arginyl residues by these ligands.

7. Microcrystal Studies

In preparation for studying the modification of crystals by

Table 3. Loss of arginyl residues in partially inactivated phosphorylase

The % activity remaining and the loss of arginyl residues were determined after exposure to varying reagent concentrations and reaction times, in the presence and absence of ligands. Arginine content determined from amino acid analysis was normalized using alanine as an internal standard. Control samples were treated identically except for the omission of butanedione and showed arginine content close to that reported by Sevilla and Fischer, 59 ± 1 arginyl residues per 92,500 g enzyme (36).

Table 3

Loss of arginyl residues in partially inactivated phosphorylases

Conditions	% Activity	Loss of arginine per 92,500 g enzyme
Phosphorylase <u>a</u> :		
5 mM butanedione, 10 min	50	4
5 mM butanedione, 20 min	37	8
5 mM butanedione, 30 min	25	9
5 mM butanedione, with 35 mM GlP, 30 min	50	7
1 mM butanedione, 20 min	71	6
2 mM butanedione, 20 min	60	7
5 mM butanedione, 20 min	37	8
Phosphorylase <u>b</u> :		
12 mM butanedione, 7.5 min	43	6
5 mM butanedione, 7.5 min	75	3
5 mM butanedione, with 1 mM AMP, 7.5 min	96	2

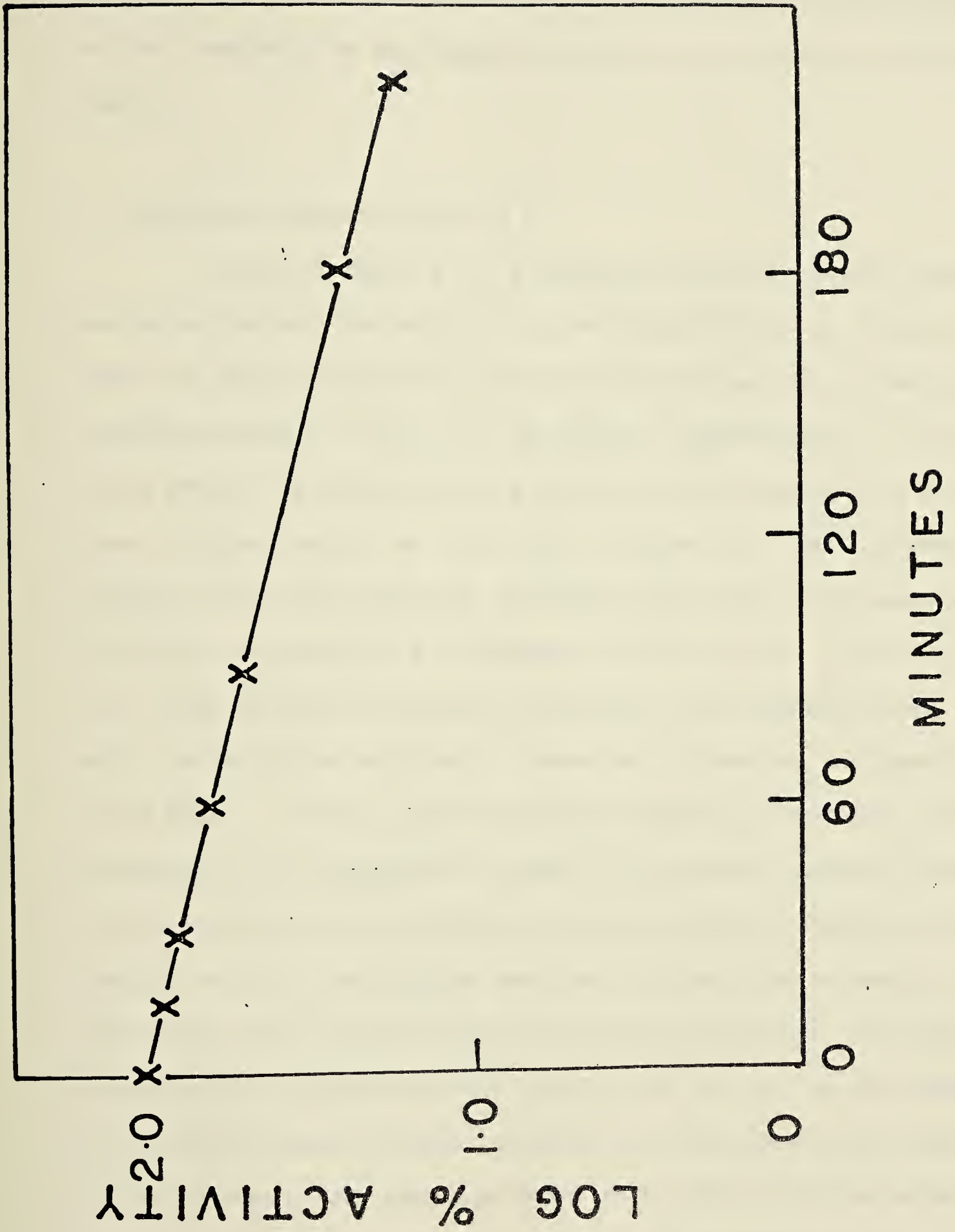
x-ray crystallography, preliminary work was carried out on glutaraldehyde cross-linked microcrystals of phosphorylase a. Typically, the activity of the native cross-linked microcrystals was 15 μ moles P_i liberated per mg enzyme, in 15 minute assays; this agrees well with the results of Kasvinsky and Madsen (17). As shown in Figure 6, first-order inactivation resulted from the reaction with a 30-fold molar excess of reagent over arginyl residues. The rate of inactivation was much slower for the microcrystals than for the enzyme in solution; the second order rate constant of inactivation was $0.7 \text{ M}^{-1} \text{ min}^{-1}$ for the reaction of 0.5 mg/ml microcrystals with 10 mM butanedione. The slower rate may reflect the effects of slower diffusion rates, different ligand affinity, cross-linking or differences in the ease of conformational changes which may be necessary for reaction and inactivation.

After 3 hours of reaction with butanedione in the absence of ligands, the microcrystals exhibited 21% activity. The microcrystals exhibited 43% activity after reaction in the presence of 1 mM AMP and 35 mM glucose-1-P, 16% activity in the presence of AMP alone, and 21% activity in the presence of glucose-1-P alone. Thus, AMP increased slightly the rate of inactivation of the microcrystals while AMP with glucose-1-P provided significant protection, results which are similar to those obtained with phosphorylase a in solution. Glucose-1-P by itself, however, had little effect; this may be a reflection of less tight binding of glucose-1-P to the microcrystals than to the enzyme in solution, or to differences in the ease or magnitude of conformational changes induced by glucose-1-P binding in the two states of enzyme.



Figure 6.

Inactivation of phosphorylase a in cross-linked micro-crystals (0.5 mg/ml) by 10 mM butanedione in 50 mM sodium borate-1 mM EDTA-5 mM DTT buffer at pH 7.5.



It is appropriate to note here that the general similarity in the behaviour of the enzyme in solution and of the microcrystals in their reaction with butanedione as well as with substrates provides evidence for the similarity of the enzyme structure in the soluble and crystalline states.

8. Difference Fourier Maps at 6 Å

Figure 7A shows a 3.0 Å resolution electron density map of the anion-binding site region of native phosphorylase a. Figure 7B shows the superposition of a difference Fourier map at 6 Å resolution, calculated using the structure amplitudes $F(\text{butanedione}) - F(\text{native})$, from a crystal of phosphorylase a which had been reacted with butanedione in borate buffer for four hours. Figure 7C is the difference electron density map using the structure amplitudes $F(\text{butanedione}) - F(\text{native})$, contoured at Z coordinates of 0.22 to 0.27, and 0.40 to 0.46, which include the glucose-binding and anion-binding sites, respectively; the molecular outlines in these two regions are indicated by the dotted lines. The root mean square difference of 4% between the structure amplitudes of the butanedione-treated and the native enzymes indicates that the reaction with butanedione did not cause very large structural changes, and that inactivation resulted from reaction at specific sites rather than from a general loss of structural integrity. The very intense peak of electron density found at the end of the side chain in the location where acetate, glucose-1-P, and arsenate are found to bind is strong presumptive evidence that this side chain is an arginyl residue. The side chain in the 3.0 Å resolution map is about 7 Å in length and has the expected shape for an arginyl residue at 3.0 Å resolution. Approximately three or four other significant

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Figure 7

A. Electron density map at 3 \AA resolution of the anion-binding site region of native phosphorylase a ($Z = 0.405$ to $Z = 0.428$). The arrow indicates the residue on which glucose-1-P and acetate are found to bind in the native crystals.

B. Difference Fourier electron density map at 6 \AA resolution using the structure amplitudes $F(\text{butanedione}) - F(\text{native})$, of a crystal of phosphorylase a soaked in 2.7 mM butanedione (in 50 mM sodium borate-1 mM EDTA-5 mM DTT at pH 7.5) superimposed on the electron density map at 3 \AA resolution of the region of the native protein shown in Figure 7A. Dashed lines are positive contours; no negative contours are seen for this section of the map. The arrow points to the most intense peak of electron density on the difference Fourier map. This corresponds to the location where arsenate, glucose-1-P, acetate and AMP are found to bind in the native crystals.

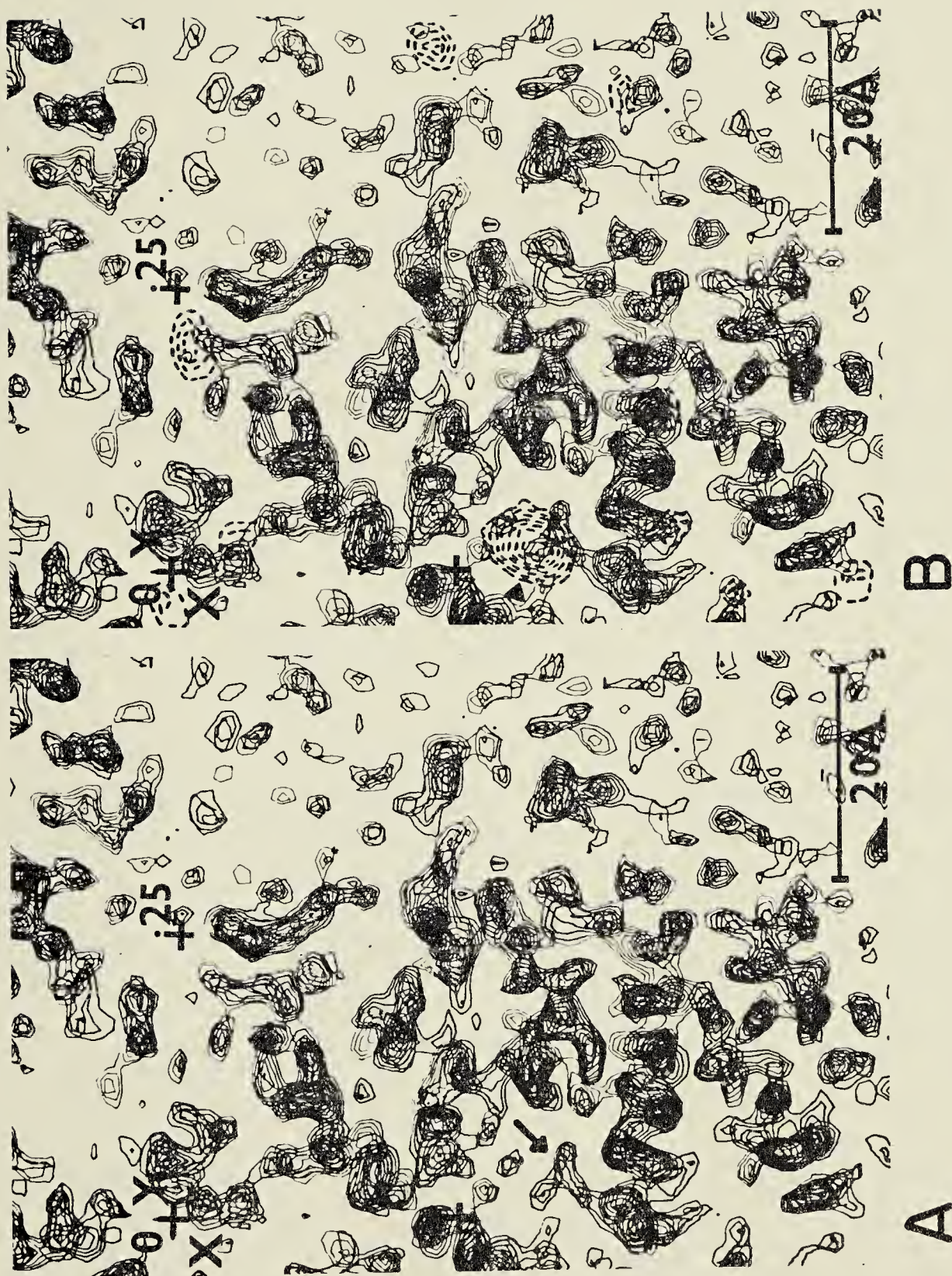


Figure 7.

C. Difference Fourier electron density map at 6 Å^o resolution using the structure amplitudes F (butanedione) - F (native). Dotted lines delineate the molecular outlines at Z coordinates of 0.40 to 0.46 and 0.22 to 0.27, which include the anion-binding and glucose-binding sites, respectively. X and Y coordinates at 0 and 0.25 are indicated by +. The horizontal bar represents 10 Å^o. Positive changes are indicated by white contour lines, negative changes by dark lines.

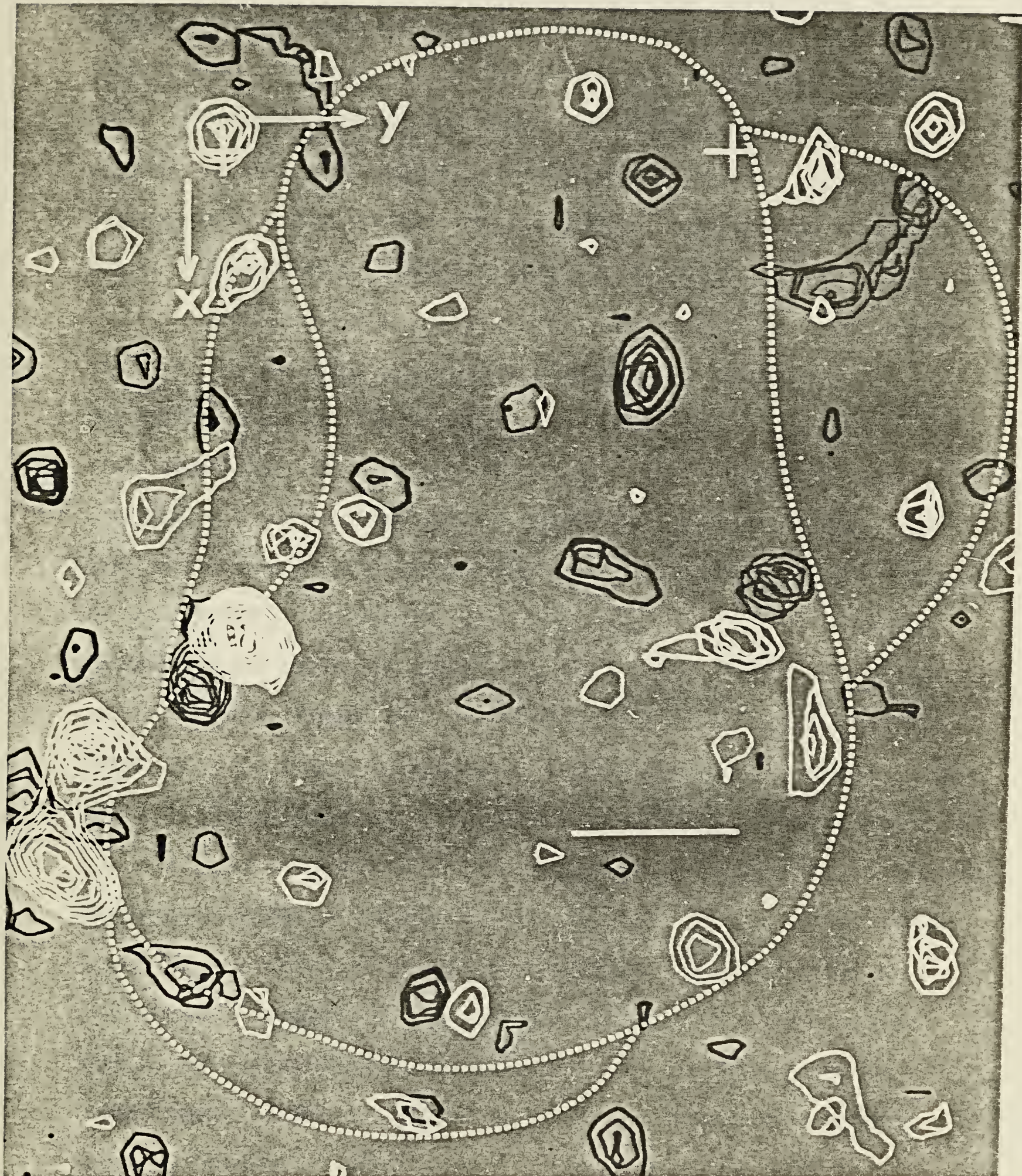


Figure 7C

electron density peaks are found on the difference Fourier map, with peak heights less than that of the intense peak at the anion-binding site. These smaller peaks are nearly all on the outer surface of the protein molecule, and together with the results of amino acid analyses, suggest that several surface-exposed arginine residues have reacted, but to a lesser degree than the residue in the anion-binding pocket.

Additional difference Fouriers were calculated to ascertain the effect of blocking this arginyl side chain on the binding of AMP and UDP-glucose. The difference Fourier map at 6 Å resolution calculated using the structure amplitudes F (cross-linked, butanedione, 1 mM AMP) - F (native) is shown in Figure 8A. The intense positive peak of electron density corresponding to the butanedione-borate complex is still found at the arginyl side chain, and the presence of the AMP molecule is not clear, in contrast to the definite binding of AMP in native crystals (Figure 8B). The difference Fourier map at 6 Å calculated using the structure amplitudes F (cross-linked, butanedione, 1 mM AMP) - F (butanedione) was compared to the difference Fourier map at 6 Å calculated using the structure amplitudes F (0.5 mM AMP) - F (native). The nucleotide AMP is found to bind in the same location in the butanedione-treated crystal as in the native crystal. However, based on peak heights, occupancy in the treated crystal is only about half of that in the native crystal, in spite of a two-fold increase in the concentration of AMP in the soaking buffer. A similar experiment to check the binding of UDP-glucose at a concentration of 50 mM to butanedione-treated crystals (Figure 9) showed that the competitive inhibitor fails to bind at the anion-binding site, but does still bind

Figure 8.

Difference Fourier maps of AMP binding to phosphorylase

A. Difference map at 6 Å resolution of the binding of 1 mM AMP to phosphorylase a crystals reacted with 2.7 mM butanedione.

B. Difference map at 4.5 Å resolution of the binding of 4 mM AMP to native crystals of phosphorylase a.

Positive changes in electron density are indicated by white contour lines, negative changes by dark lines. Dotted lines delineate the molecular outlines at the 2 regions along Z that include the anion-binding and glucose-binding sites, which are at Z coordinates of 0.40 to 0.46, and 0.22 to 0.27, respectively, in A, and 0.36 to 0.44, and 0.20 to 0.28, respectively, in B. X and Y coordinates at 0 and 0.25 are indicated by +. The horizontal bar represents 10 Å.

A = phosphate moiety.



Figure 8A



Figure 8B

Figure 9.

Difference Fourier maps of UDP-glucose binding to phosphorylase a.

A. Difference map at 6 Å resolution of the binding of 50 mM UDP-glucose to phosphorylase a crystals reacted with 2.7 mM butanedione.

B. Difference map at 4.5 Å resolution of the binding of 20 mM UDP-glucose to native crystals of phosphorylase a.

Symbols and X, Y, and Z coordinates are as described in the legend to Figure 8.

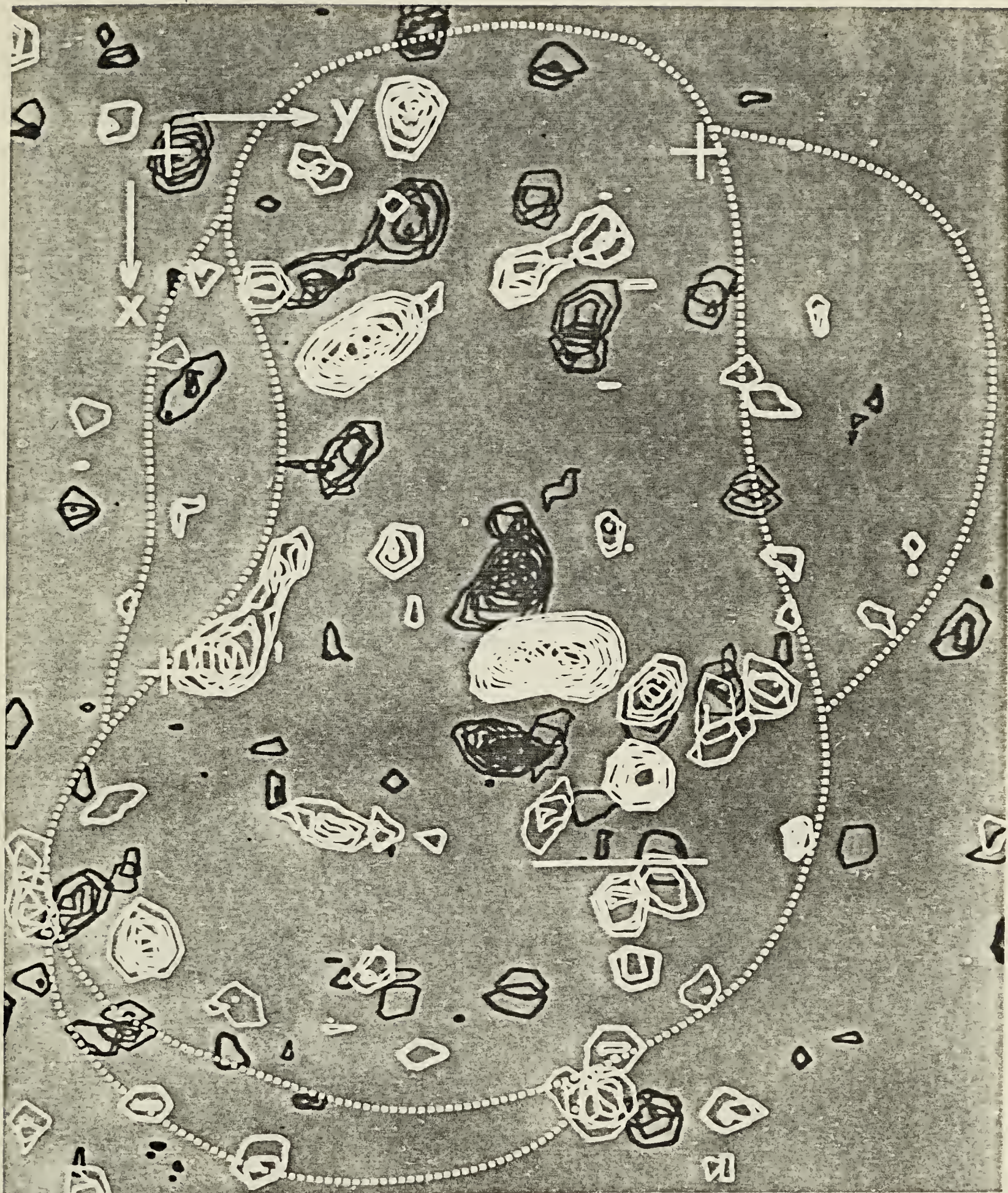


Figure 9A



Figure 9B

to the site in the NAD-type binding domain at which glucose (50 mM), glucose-1-P (300 mM) and UDP-glucose (20 mM) bind in the native enzyme. Structural changes accompanied this binding, and a root mean square difference of 16% was obtained for the structure amplitudes. $F(\text{butanedione, 50 mM UDP-glucose}) - F(\text{native})$, compared to a value of 10% for $F(20 \text{ mM UDP-glucose}) - F(\text{native})$. These results are consistent with the protection studies carried out with the solution and microcrystalline forms of the enzyme.

C. Discussion

The present work indicates the involvement of an essential arginyl residue in glycogen phosphorylase. The selectivity of 2,3-butanedione in sodium borate buffer for arginyl residues in other proteins has been well documented (31), and is supported in the present study by the pH dependence of the reaction, ability to quench the reaction with free arginine, and amino acid analysis results. The ability to reverse the reaction and partially restore enzymic activity is also important in demonstrating the absence of unsuspected irreversible damage to the enzyme. Ultracentrifugal analysis also eliminates the possible involvement of association or dissociation as the cause of inactivation.

The results of crystallographic studies suggest that the location of the inactivating reaction is an anion-binding site. A 3.0 Å resolution electron density map of this region in phosphorylase a has been recently published (16), from which can be seen a long side chain in the pocket formed by a V-shaped framework of two α-

helices. A series of difference Fourier maps derived from crystals soaked in various ligands indicates that glucose-1-P and arsenate (an alternative substrate) both bind at the end of this side chain, as do parts of the allosteric effector AMP, the competitive inhibitor UDP-glucose, as well as many anions such as acetate. The present study provides strong evidence identifying this long chain as the guanidinium side chain of an arginyl residue, and indicates that inactivation of phosphorylase by butanedione is primarily the result of reaction of this side chain.

The retardation of loss of activity by the substrate glucose-1-P, alone or together with AMP, as well as by the competitive inhibitor UDP-glucose, and the order of reaction with respect to butanedione concentration both support the view that the decreased activity in phosphorylase a results from modification of an arginyl residue at specific ligand binding sites. Further confirmation is supplied by the appearance of increased electron density at the long side chain at the anion-binding site pocket of butanedione-treated crystals. AMP was found to bind in the butanedione-treated crystals in the same position as in the native enzyme, but with less occupancy. Since microcrystal studies had indicated incomplete inactivation under similar reaction conditions, the binding in the treated crystals may reflect occupancy at the AMP sites of unreacted enzyme. Alternatively, a displacement of the equilibrium between free and reacted enzyme, (enzyme-arginine-butanedione $\xrightleftharpoons{\rightarrow}$ enzyme-arginine + butanedione), may result from the presence of AMP to favor the binding of AMP to the free enzyme (enzyme + AMP $\xrightleftharpoons{\rightarrow}$ enzyme-AMP). This might be expected if

the site of butanedione reaction is at the AMP binding site or at the neighboring glucose-1-P binding site. This hypothesis was also proposed by Marcus (30), who observed that the presence of AMP favors the reversal of butanedione modification at the AMP site of fructose-1,6-bisphosphatase. However, the possibility of a different and less favorable mode of binding in modified enzyme cannot be excluded. The slight enhancement of inactivation rate by AMP in both microcrystals and solution forms of phosphorylase a may be due to conformational changes enhancing affinity for the rather bulky butanedione-borate complex at the arginyl residue. The protection by AMP in the solution form of phosphorylase b, however, contrasts with the behaviour in phosphorylase a, and points out that subtle differences must exist in the binding of AMP to phosphorylases a and b.

While there is little doubt regarding the importance of the arginyl residue, several observations make it unlikely that this residue is directly involved in catalysis. Firstly, the fact that butanedione-treated phosphorylase b exhibits an increase in AMP-independent activity suggests that the site of butanedione modification is related to the AMP-binding site rather than the site of catalysis. Furthermore, the difference Fourier map for the binding of UDP-glucose to the modified crystals indicates the absence of UDP-glucose at the anion-binding site but strong binding at the glucose-binding locus in the NAD-type binding domain, whereas in the native crystals, UDP-glucose binds at both of these sites.

Kinetic studies suggest that an essential activation precedes actual catalysis (37). If it is assumed that the anion-binding site

is the essential activator site, then a chemical modification at the anion-binding site may not prevent the binding of substrates at a spatially distant active site. However, the resulting modified enzyme-substrate complex might be expected to be nonproductive or to exhibit only poor activity since the normal prerequisite activation has been prevented. Although the present study does not present unequivocal proof for this model, the findings are consistent, the site of modification being the activator site, and the site at which UDP-glucose still binds being the active site. Protection of the activator site from modification by glucose-1-P and UDP-glucose may be explained by a direct blocking of the activator site, or by indirect effects transmitted on binding at the active site, while the effects of AMP would be probably directly at the activator site.

Chapter IV

Modification with Potassium Ferrate

A. Introduction

Many of the ligands important in the control and activity of phosphorylase contain a phosphate group - for example, the substrates glucose-1-P and phosphate, competitive inhibitor UDP-glucose, and allosteric activators and inhibitors such as AMP, IMP, ATP and glucose-6-P. Thus one would expect that a reagent which is also a phosphate analogue would be potentially very useful in the study of this enzyme. Potassium ferrate is such a reagent. Ferrate ion in aqueous solution as well as in crystalline form possesses tetrahedral symmetry (38, 39) and exists either as the dianion or monoanion under neutral pH conditions. The iron-oxygen bond length has been shown by crystallography to be 1.656 Å (39), in contrast to 1.56 Å for the phosphorous-oxygen bond in orthophosphate (40). However, in addition to these properties, ferrate is a very strong oxidizing agent capable of oxidizing a variety of functional groups. For example, primary alcohols and amines and secondary alcohols are oxidized rapidly to the corresponding aldehydes and ketones (41).

The reaction of rabbit muscle phosphorylase b with ferrate was studied by Lee and Benisek (15). Inactivation of the enzyme was accompanied by loss of AMP binding and the loss of 1 or 2 tyrosyl residues and 1 cysteinyl residue. Both activating and non-activating nucleotides such as 5'-AMP, 2'-AMP, 3'-AMP and IMP provided substantial protection from inactivation, presumably by protection of 1 or 2 tyrosyl residues.

These results were proposed to indicate that tyrosine modification was the inactivating reaction and that the site of inactivation was at or near the AMP binding site. However, protection from inactivation was afforded also by other ligands such as glucose-1-P, glycerophosphate and acetate. The degree of this protection varied with increasing concentration of the ligands, protection appearing to be a "saturable" phenomenon, suggesting, perhaps, protection by a direct blocking of the site of modification. No convincing explanation could be given for these observations if inactivation occurred as a result of modification at a site exclusively binding AMP and AMP analogues. Since these authors limited their work to the reaction with phosphorylase b only, it seemed worthwhile to carry out parallel studies in phosphorylase a, and further to confirm the site and effects of inactivation using x-ray crystallography.

B. Results

1. Phosphorylase b

The inactivation of 30 μ M phosphorylase b with varying molar excesses of ferrate is shown in Figure 10. These results are similar to those obtained by Lee and Benisek (15), if one takes into account the different extinction coefficient used by these authors for calculating enzyme concentration ($E_{1\text{cm}}^{1\%}$ at 280 nm = 11.9, compared to a value of 13.2 used in our studies). Extrapolation of the linear portion in Figure 10 indicates complete inactivation at about 10 moles ferrate

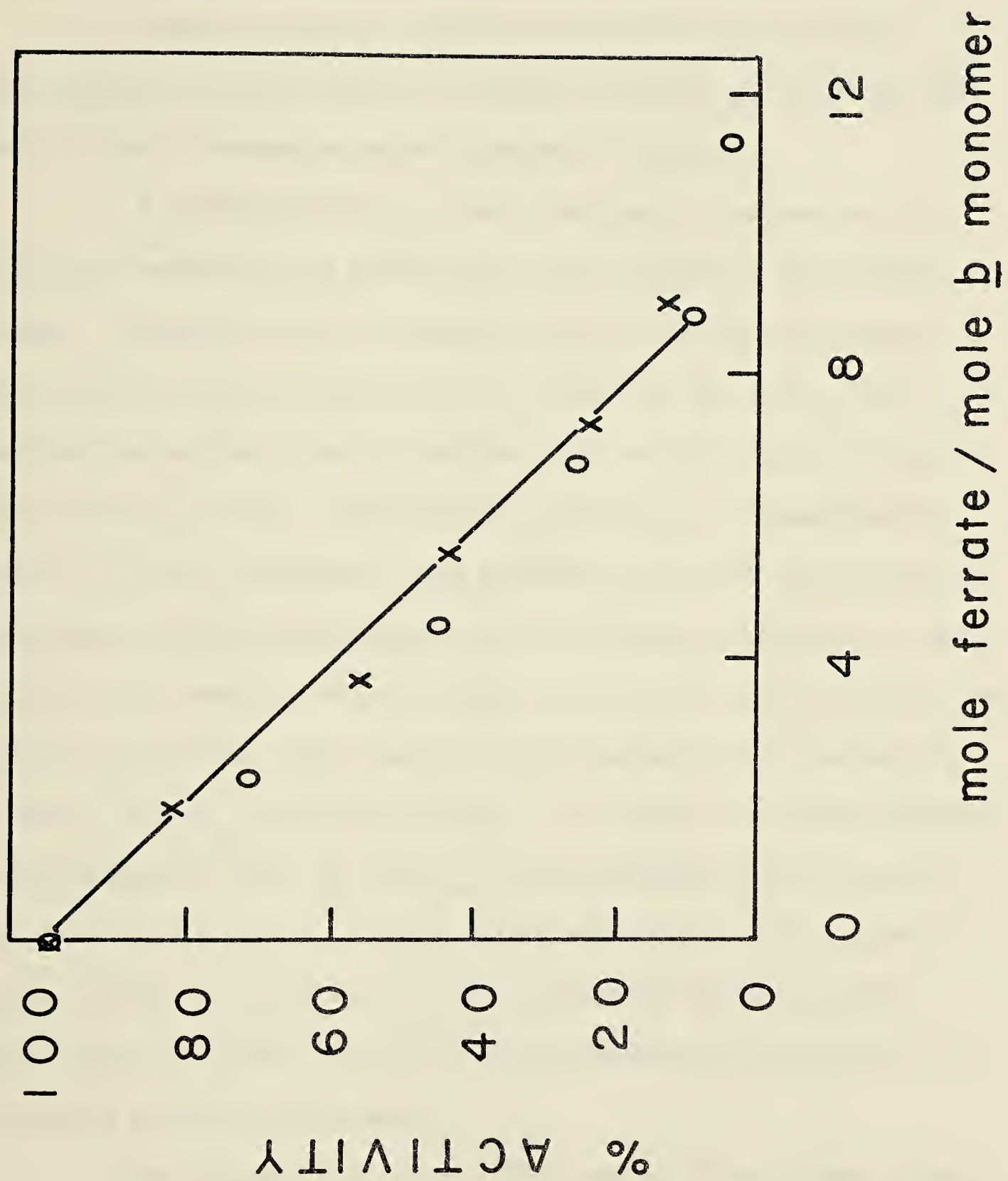


Figure 10.

Inactivation of phosphorylase b as a function of increasing ferrate:enzyme molar ratio.

Aliquots of 3 mM ferrate in 0.1 mM NaOH were added to 30 μ M phosphorylase b in 5 mM Na cacodylate buffer at pH 7.0 until the indicated molar ratios were attained.

- X experimental data from the present study
- 0 data from Lee and Benisek's work (15), after adjustments using $E_{1\text{cm}}^{1\%}$ of 13.2 instead of 11.9.



per mole b monomer. However, this value does not indicate the true stoichiometry of inactivation since ferrate may react with the buffer components and water as well as with protein.

Assays of enzyme activity carried out in the absence of AMP indicated little change or a slight decrease in the minus AMP activity with increasing molar excesses of ferrate.

Lineweaver Burk plots were obtained to determine the Michaelis constants for substrates in the partially inactivated enzyme. Figures 11 and 12 indicate that in the case of varying glucose-1-P, the plots intersect at a point to the left of the ordinate axis; the plots for varying AMP, on the contrary, do not intersect at a point. The Michaelis constants are summarized in Table 4. The K_m values for both glucose-1-P and AMP increase with increasing ferrate concentration and increasing inactivation, about 3-fold after reaction with 4.5 moles ferrate per mole b monomer, and about 6- to 7-fold after reaction with 9 moles ferrate per mole b monomer. The K_m value for glycogen, in contrast, decreased slightly. These changes in both K_m and V_{max} values indicate that during the course of modification, various intermediate species of enzyme may appear which still retain catalytic properties but may possess a modified or decreased ability to bind substrates or activator or a decreased catalytic efficiency.

The change in K_m value of AMP may at first glance appear anomalous since Lee and Benisek (15) showed by gel filtration that inactivated enzyme showed no detectable binding of AMP. However, since the characteristics of only partially inactivated enzyme are being

Table 4

Kinetic parameters for phosphorylase b partially inactivated by ferrate

Mole ferrate per mole monomer ^a	Varying GLP ^b		Varying AMP ^c		Varying Glycogen ^d	
	K _m	V _{max}	K _m	V _{max}	K _m	V _{max}
0	2.5	64.6	0.022	60.9	0.056	61.9
4.5	8.0	38.9	0.078	32.0	0.040	31.2
9.0	15.4	19.6	0.161	15.3	0.027	12.7

NOTE: Units for V_{max} are μmoles/min/mg at 30°, and for K_m are millimolar for GLP and AMP, and percent for glycogen.

^aPhosphorylase b at a concentration of 30 μM was treated with ferrate to give the indicated molar ratios of ferrate per monomer of phosphorylase b, prior to determination of enzymatic activity.

^bdetermined at 1% glycogen, 1 mM AMP

^cdetermined at 1% glycogen, 24 mM GLP

^ddetermined at 24 mM GLP, 1 mM AMP



Figure 11.

Activity of ferrate-treated phosphorylase b as a function of glucose-1-P concentration.

Phosphorylase b (30 μ M) was reacted with the following molar excesses of ferrate: **●**, no ferrate; X, 4.5 moles ferrate per mole b monomer; **○**, 9 moles ferrate per mole b monomer. Assays contained 1 mM AMP, 1% glycogen and varying concentrations of glucose-1-P.

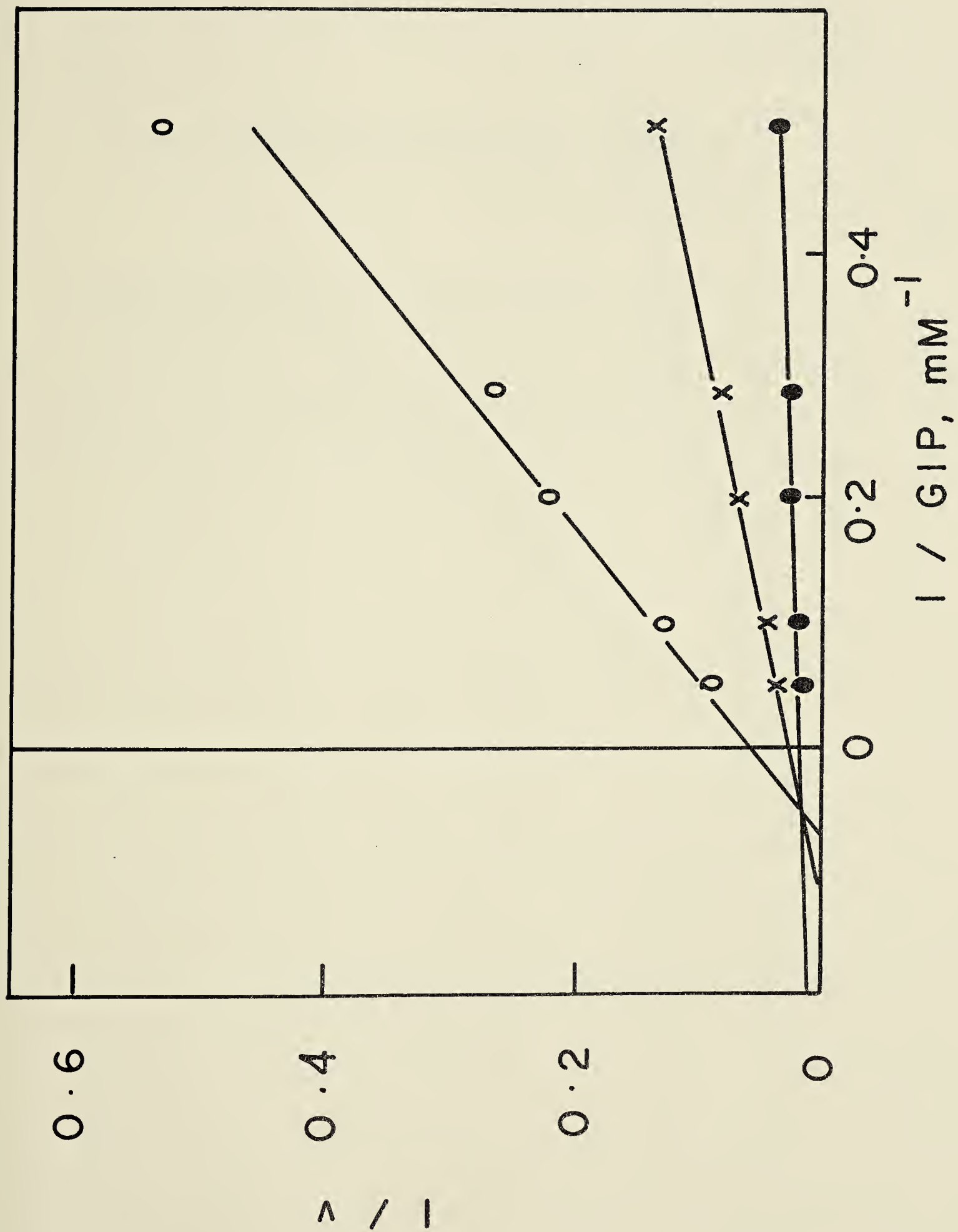




Figure 12.

Activity of ferrate-treated phosphorylase b as a function of AMP concentration.

Phosphorylase b (30 μ M) was reacted with the following molar excesses of ferrate: \bullet , no ferrate; X, 4.5 moles ferrate per mole b monomer; O, 9 moles ferrate per mole b monomer. Assays contained 24 mM glucose-1-P, 1% glycogen and varying concentrations of AMP.

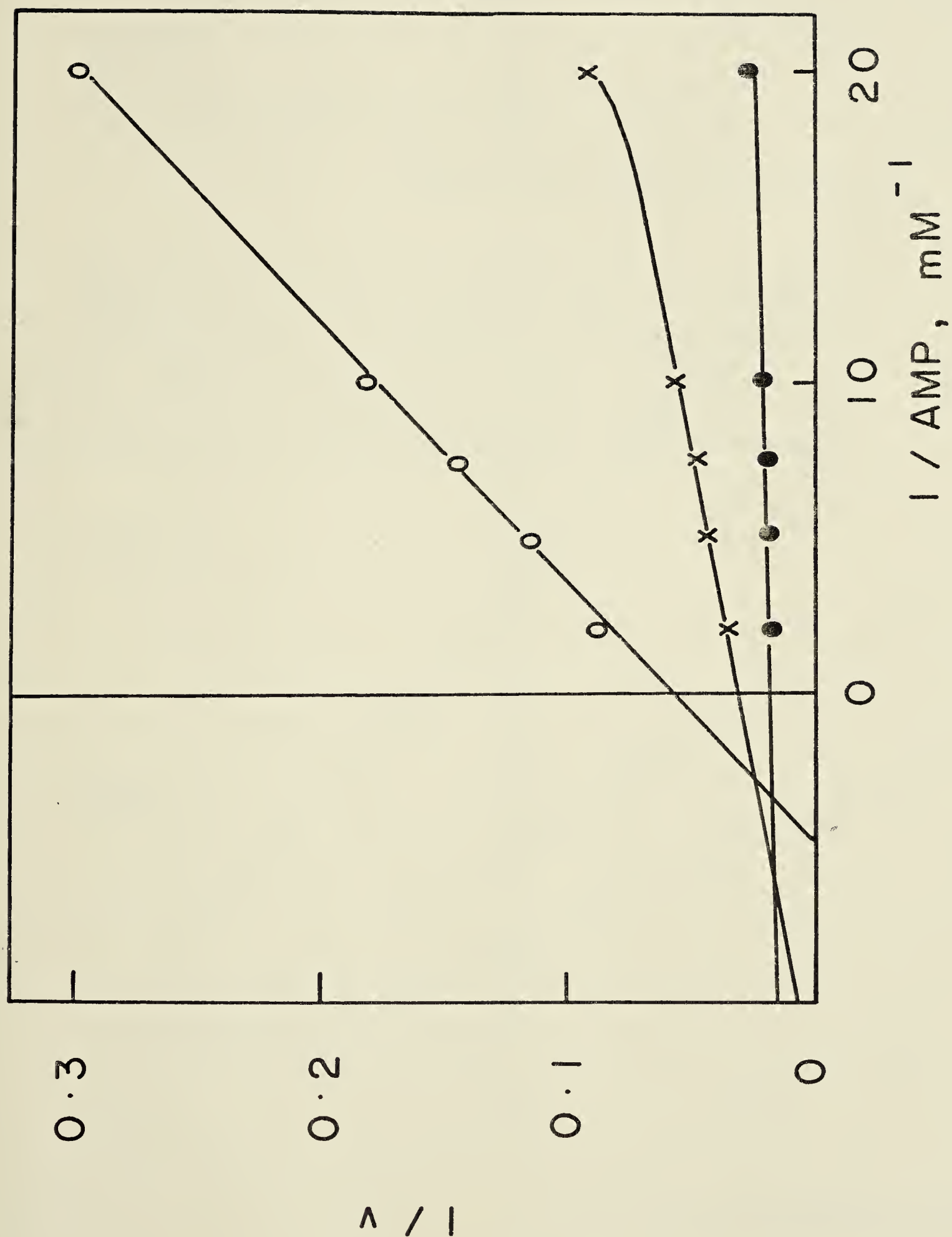
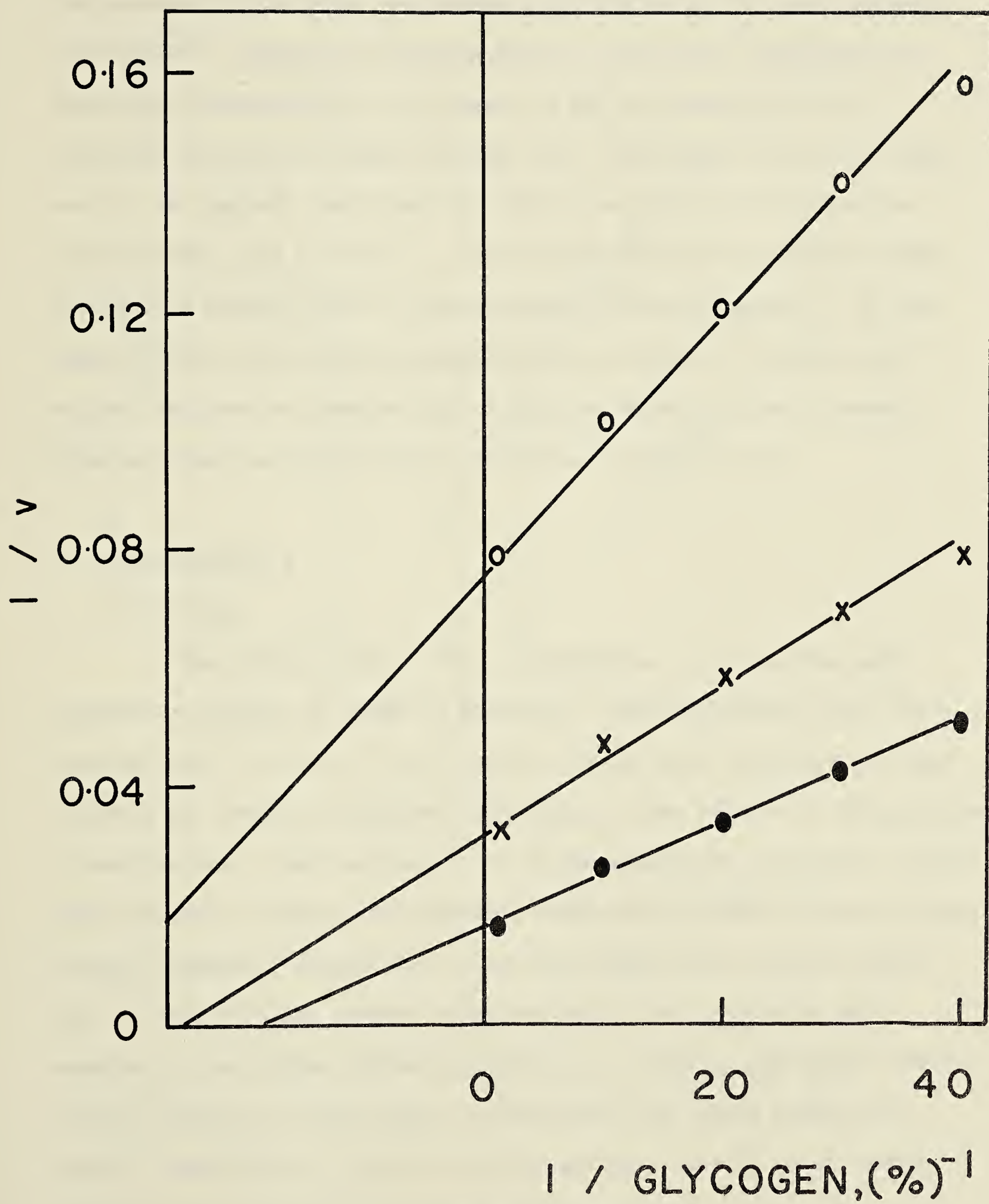




Figure 13.

Activity of ferrate-treated phosphorylase b as a function of glycogen concentration.

Phosphorylase b (30 μ M) was reacted with the following molar excesses of ferrate: **●**, no ferrate; **X**, 4.5 moles ferrate per mole b monomer; **○**, 9 moles ferrate per mole b monomer. Assays contained 1 mM AMP, 24 mM glucose-1-P and varying concentrations of glycogen.



studied in this case, it is possible that modification of only one of the subunits in the dimer may change the affinity of the other subunit for ligands. Analysis of the same data on a Hill plot indicates that homotropic cooperativity with respect to AMP was decreased in the partially inactivated enzyme (Figure 14). The slopes of the Hill plots are 1.0 at high AMP concentrations and 1.6 at low concentrations for native enzyme, and 1.0 and 1.3 respectively for 47% inactivated enzyme, obtained by reaction with 4.5 moles ferrate per mole monomer. In the range of AMP concentration studied (0.05 to 0.5 mM), 75% inactivated enzyme, obtained by reaction with 9.0 moles ferrate per mole monomer, shows no break in its Hill plot, which has a slope of 1.0.

2. Phosphorylase a

a. In Solution

The inactivation of 30 μ M phosphorylase a by varying molar excesses of ferrate is shown in Figure 15. Extrapolation of the linear portion gives a value of 9 or 10 moles ferrate per mole monomer phosphorylase a for complete inactivation, similar to the results of phosphorylase b inactivation. The reaction with 1 μ M phosphorylase a required a larger molar excess of reagent for complete inactivation, about 20 moles ferrate per mole monomer a (Figure 15). This may suggest that ferrate reacts more readily with the enzyme in its tetrameric form, which is more abundant at the higher enzyme concentration. However, the greater molar excess required at lower enzyme concentration may simply reflect the greater instability of dilute ferrate solutions, as well as the effect of dilution on the rate of a bimolecular reaction.

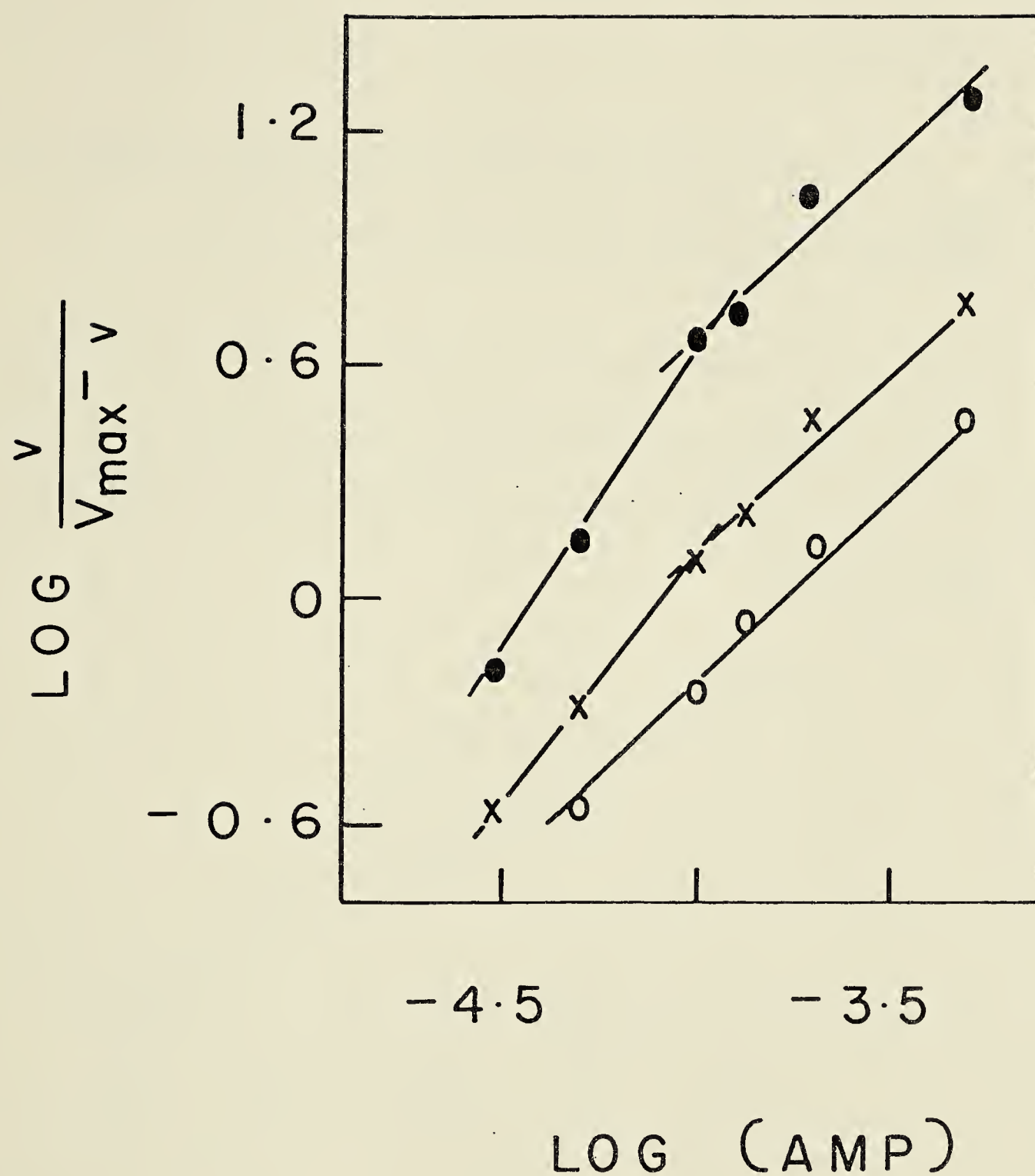


Figure 14.

Hill plot of the data from Figure 12, showing the effect of ferrate modification on the activity of phosphorylase b at varying AMP concentrations.

- no ferrate
- X 4.5 moles ferrate per mole b monomer
- O 9 moles ferrate per mole b monomer



Figure 15.

Inactivation of 1 μ M or 30 μ M phosphorylase a as a function of increasing ferrate:enzyme molar ratio.

Aliquots of 1 mM or 3 mM ferrate in 0.1 mM NaOH were added to 1 (X) or 30 (O) μ M phosphorylase a, respectively, in 5 mM Na cacodylate buffer at pH 7.0, until the indicated molar ratios were attained.

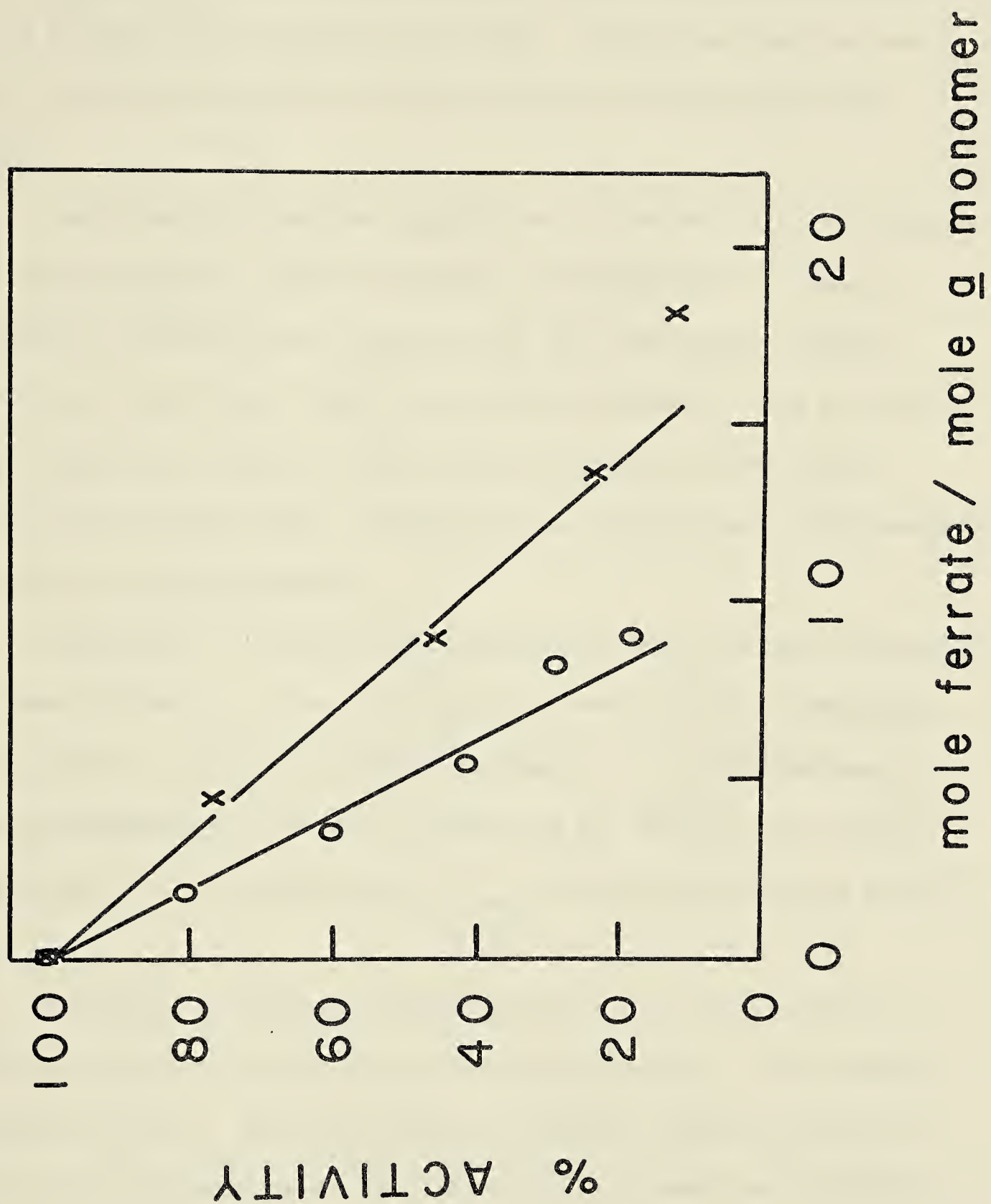


Figure 16 shows that the reaction was essentially complete within 10 seconds after addition of ferrate. Since the reaction was so rapid, no detailed studies on the kinetics of inactivation were attempted.

The effects of various ligands on the extent of inactivation of 1 μ M phosphorylase a by 20 μ M ferrate are summarized in Table 5. Some degree of protection was exhibited by all the ligands tested, although that of AMP and of MgOAc were rather minimal. Good protection occurred with AMP and glucose-1-P or AMP and glucose; even better protection resulted when AMP, glucose-1-P and glucose were simultaneously present in the reaction mixture.

The effect of ferrate inactivation of the K_m value of glucose-1-P is shown in Table 6. The increase in K_m was smaller in magnitude than that found in the case of phosphorylase b. A 7-fold decrease in V_{max} was accompanied by a 4-fold increase in K_m , while in the case of phosphorylase b, a 3-fold decrease in V_{max} was accompanied by a 6-fold increase in K_m .

Partially inactivated phosphorylase a was checked for its response to activation by AMP and inhibition by glucose. The results, summarized in Table 7, show that ferrate-modified enzyme possessed a slightly greater dependence on AMP for activity as well as a slightly greater inhibition by glucose. The presence of either glucose-1-P or UDP-glucose during the reaction with ferrate decreased the extent of inactivation and prevented the change in AMP-dependence for activity (Table 8).

Amino acid analyses of partially inactivated phosphorylase a were also performed (Table 9). The reaction of 30 μ M phosphorylase a

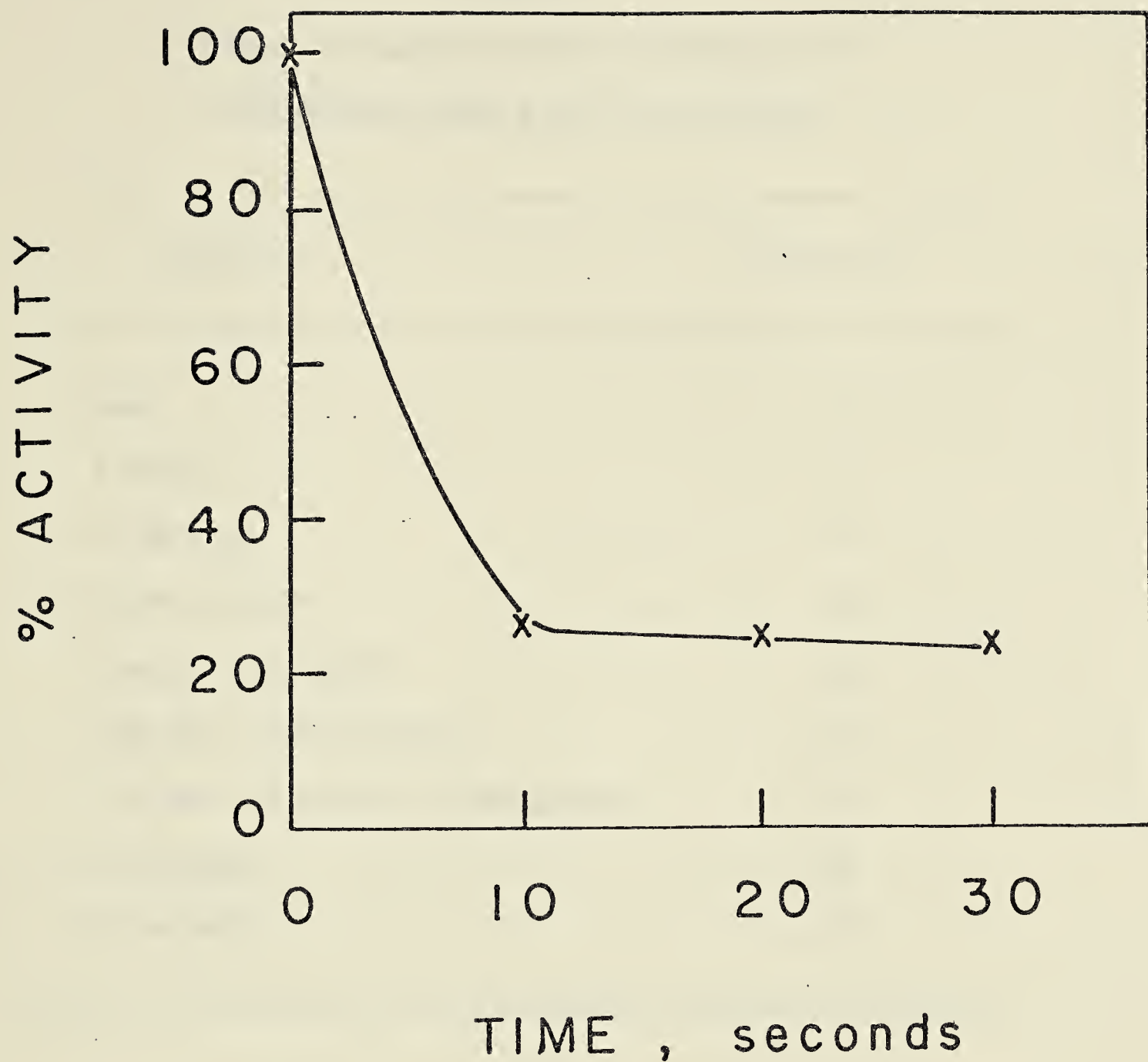


Figure 16.

Inactivation of 1 μM phosphorylase a by 14 μM ferrate as a function of time. The reactions were terminated at the desired time by addition of assay buffer containing 2-mercaptoethanol at a final concentration of 10 mM.

Table 5
Effect of ligands on the inactivation of
1 μ M phosphorylase a by 20 μ M ferrate

Ligand(s)	% Activity
none	13
1 mM AMP	21
26 mM G1P	39
50 mM glucose	32
1 mM AMP, 26 mM G1P	60
1 mM AMP, 50 mM glucose	51
1 mM AMP, 26 mM G1P, 50 mM glucose	73
1% glycogen	39
50 mM MgOAc	26

Phosphorylase a at a concentration of 1 μ M was pre-incubated with the indicated ligand(s) at room temperature, prior to addition of ferrate to a final concentration of 20 μ M.

Table 6

Kinetic Parameters for

phosphorylase a partially inactivated by ferrate

Mole ferrate per mole <u>a</u> monomer	Varying GlP	
	K_m , mM	V_{max} , $\mu\text{mole}/\text{min}/\text{mg}$
0	0.4 ± 0.1	60.8 ± 1.2
9	0.7 ± 0.1	27.8 ± 0.5
18	1.7 ± 0.2	9.1 ± 0.3

Phosphorylase a at a concentration of 1 μM was treated with ferrate to give the indicated molar ratios of ferrate per monomer of phosphorylase a, prior to determination of enzymatic activity at varying GlP concentrations in the presence of 1% glycogen.

Table 7

Effect of ferrate reaction on the response of phosphorylase a
to AMP activation and glucose inhibition

% Activity ^a		Ratio of Activity ^b		
		<u>- AMP</u>	<u>+ glucose</u>	<u>+ AMP + glucose</u>
		+ AMP	- glucose	+ AMP - glucose
Control	100	0.76	0.55	0.90
Ferrate	30	0.67	0.45	0.80

^adetermined with 75 mM G1P, 1% glycogen, 1 mM AMP

^ball assays contained 15 mM G1P and 0.2% glycogen in the presence or absence of 1 mM AMP and in the presence or absence of 50 mM glucose

Phosphorylase a at a concentration of 10 μ M was treated with 150 μ M ferrate prior to determination of enzymatic activity.

Table 8

Effect of protecting ligands on the response of
ferrate-reacted phosphorylase a to AMP activation

	% Activity ^a	Activity ratio, $\frac{- \text{AMP}}{+ \text{AMP}}$ ^b
Control	100	0.77
Ferrate	35	0.72
Control with 30 mM G1P	100	0.78
Ferrate with 30 mM G1P	70	0.80
Control with 5 mM UDPG	100	0.75
Ferrate with 5 mM UDPG	55	0.75

^adetermined with 75 mM G1P, 1% glycogen, 1 mM AMP

^bassays contained 15 mM G1P and 1% glycogen in the presence or
absence of 1 mM AMP

Phosphorylase a at a concentration of 10 μ M was pre-incubated with
the indicated ligand prior to reaction with 150 μ M ferrate and
subsequent determination of enzymatic activity.

Table 9

Amino acid composition of untreated and ferrate-treated
phosphorylase a

	moles per 92,500 g enzyme					
	Literature ^a		Present study			
	Ref 1	Ref 2	Control	Ferrate ^b	Ferrate ^c AMP	Ferrate ^d G1P
% Activity	-	-	100	11	12	38
asp	90.83±1.30	92.16	91.0	93.8	94.5	93.5
thr	31.65±0.18	33.37	34.0	34.4	34.0	34.0
ser	27.34±0.75	26.12	25.3	26.8	25.2	24.4
glu	94.38±1.12	93.8	94.7	94.8	93.7	94.3
pro	39.32±0	39.13	36.0	37.5	38.0	39.1
gly	45.88±0.18	46	46.5	47.4	48.8	47.3
ala	60.30±0	61.41	60.3	60.3	60.3	60.3
val	56.74±0.56	58.69	59.0	57.0	56.9	59.1
met	20.22±0.37	20.67	20.9	21.0	20.1	18.3
ileu	44.94±0.38	43.69	42.1	42.1	42.2	42.9
leu	75.28±0.37	78.03	78.2	78.0	77.0	77.9
tyr	33.90±0.18	34.53	37.0	33.5	34.6	36.3
phe	35.58±0.37	36.22	37.0	36.4	36.8	36.8
his	19.29±0.19	20.35	20.5	20.6	20.5	21.0
lys	43.44±0	45.64	47.8	47.4	47.0	48.5
arg	58.98±0.94	60.17	59.6	58.9	59.9	59.9
half-cys	7.86±0.5	8.30	-	- ^e	- ^e	- ^e

^aRef 1 = Sevilla and Fischer (36); Ref 2 = Lee and Benisek (15)

^b30 µM phosphorylase a was reacted with 420 µM ferrate at 22°

^c30 µM phosphorylase a was reacted with 420 µM ferrate at 22° in the presence of 1 mM AMP

^d30 µM phosphorylase a was reacted with 420 µM ferrate at 22° in the presence of 25 mM G1P

^eformation of approximately 2 cysteic acid residues

with a 14-fold molar excess of ferrate resulted in enzyme exhibiting 11% activity and a loss of 3 to 4 tyrosyl residues per monomer, compared to the control containing 37 tyrosyl residues per monomer. When the reaction was carried out in the presence of 1 mM AMP, only 2 to 3 tyrosyl residues reacted, but activity remained at only 12%. Phosphorylase a reacted in the presence of 25 mM glucose-1-P showed a loss of less than 1 tyrosyl residue and retained activity which was 38% that of the control. The reaction was accompanied by the production of about two cysteic acid residues per monomer, unaffected by the presence of AMP or glucose-1-P.

b. In Microcrystals and Crystals

The reaction of 20 μ M ferrate with 1 μ M cross-linked microcrystals of phosphorylase a was studied as a function of time. It was anticipated that the inactivation rate would be slower than in solution (compare Figure 16), due to diffusion effects and restrictions on conformational mobility. The microcrystals exhibited 25% activity after 10 minutes reaction time, and 12% activity after 30 minutes. When the reaction was carried out in the presence of 50 mM glucose-1-P, the enzyme retained 73% activity even after 30 minutes reaction.

A root mean square difference of 3% was obtained between the structure amplitudes of ferrate-treated and native crystals of phosphorylase a, indicating that no gross changes had occurred. No distinct differences were seen in the difference Fourier map at 4.5 Å resolution calculated using the amplitudes $F(\text{ferrate}) - F(\text{native})$. The difference Fourier map calculated for $F(\text{ferrate, 1 mM AMP}) - F(\text{native})$ indicated

that AMP still bound to the activator site in the ferrate-treated enzyme (Figure 17), but peak heights showed that occupancy was only half of that in the native enzyme. The root mean square difference between the structure amplitudes was 5%, compared to a value of 9% for $F(4 \text{ mM AMP}) - F(\text{native})$. The corresponding difference Fourier for glucose-1-P binding (Figure 18) indicated that at a concentration of 50 mM, this substrate bound predominantly at the glucose-binding locus in the NAD-type binding domain. A root mean square difference of 13% was observed, compared to a value of 11% for binding of 300 mM glucose-1-P to native enzyme. The binding of 50 mM glucose-1-P to the glucose-binding locus of ferrate-treated enzyme is in contrast to native enzyme, in which the major binding locus for 50 mM glucose-1-P is the anion-binding site, a concentration of 300 mM being required to favor the binding at the glucose-binding locus.

C. Discussion

Both phosphorylases a and b are rapidly inactivated by the oxidation with the phosphate analogue, ferrate. The results of amino acid analysis of ferrate-treated phosphorylase a indicate the disappearance of tyrosyl residues, analogous to the case of phosphorylase b studied by Lee and Benisek (15). Several other workers have also implicated the role of tyrosyl residues at or near the AMP-binding site of phosphorylase. Shimomura and Fukui (42) studied the interaction of phosphorylase b with the allosteric activator AMP and its analogues by circular dichroism and suggested that a stacking interaction is formed

Figure 17.

Difference Fourier map at 4.5 Å resolution of the binding of 1 mM AMP to ferrate-treated crystals of phosphor^oylase a.

Positive changes in electron density are indicated by white contour lines, negative changes by dark lines. Dotted lines delineate the molecular outlines at Z coordinates of 0.40 to 0.46, and 0.22 to 0.27, which include the anion-binding and glucose-binding sites, respectively. X and Y coordinates at 0 and 0.25 are indicated by +. The horizontal bar represents 10 Å.^o

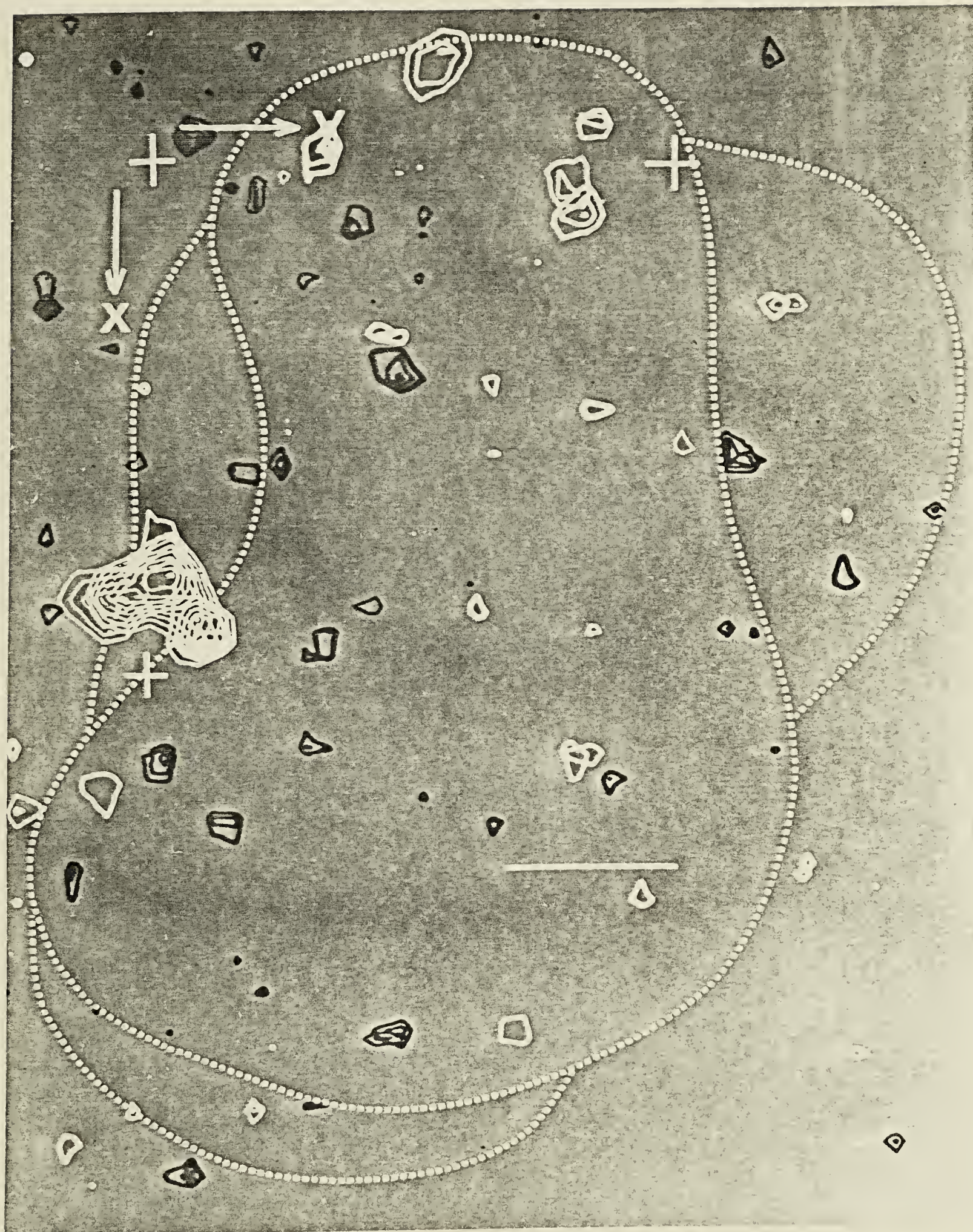


Figure 17

Figure 18.

Difference Fourier maps at 4.5 Å resolution of the glucose-1-P binding loci in native and ferrate-treated crystals of phosphorylase a.

- A. Binding of 50 mM glucose-1-P to ferrate-treated phosphorylase a.
- B. Binding of 50 mM glucose-1-P to native phosphorylase a.
- C. Binding of 300 mM glucose-1-P to native phosphorylase a.

Symbols are as described in the legend to Figure 17. The Z coordinates are 0.40 to 0.46, and 0.22 to 0.27 for A, and 0.36 to 0.44, and 0.20 to 0.28 for B and C.

A = phosphate moiety; GlP = glucose and phosphate moieties at the site for free glucose (G) and the adjacent site for the phosphate (1P) moiety of glucose-1-P.

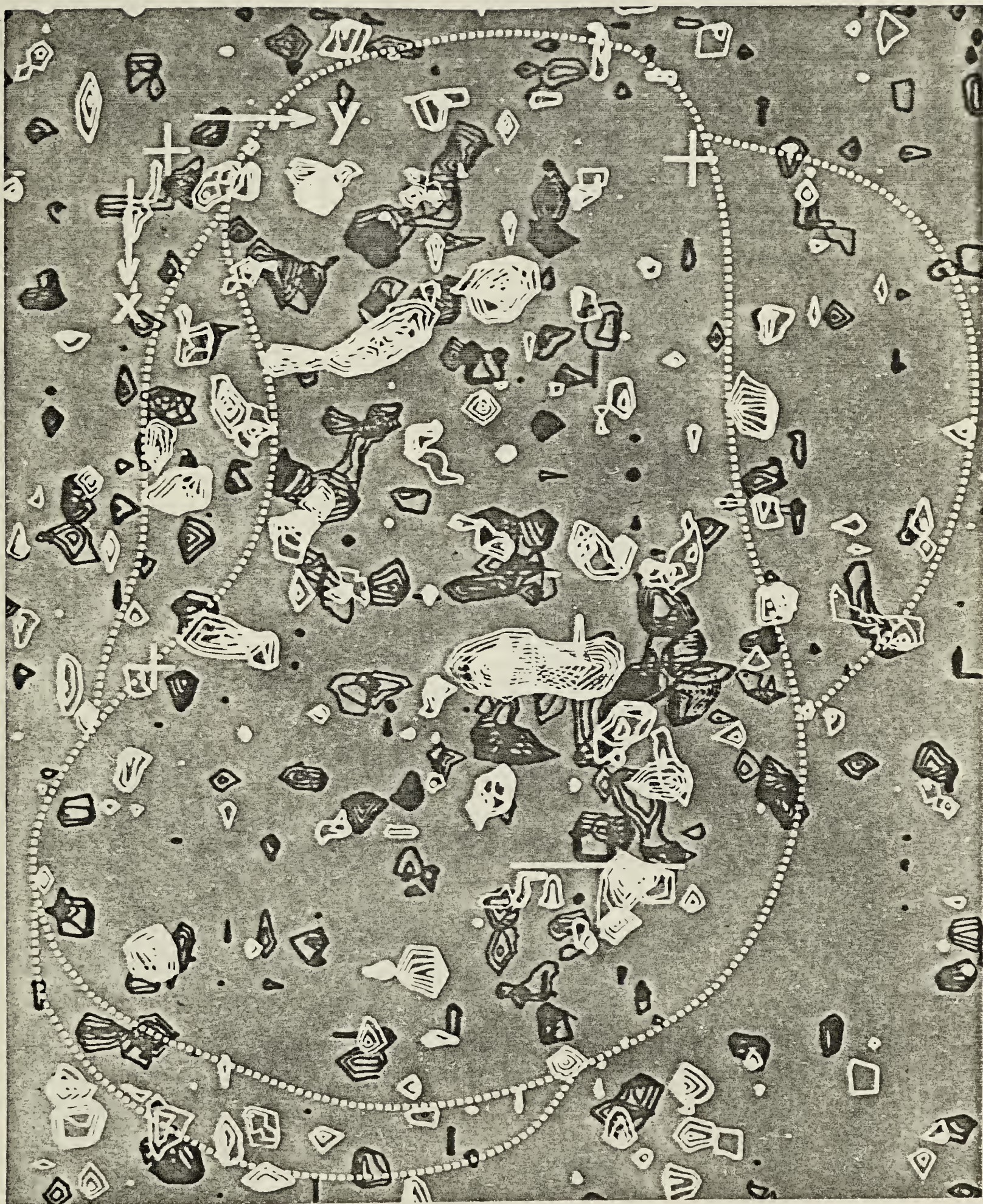


Figure 18A

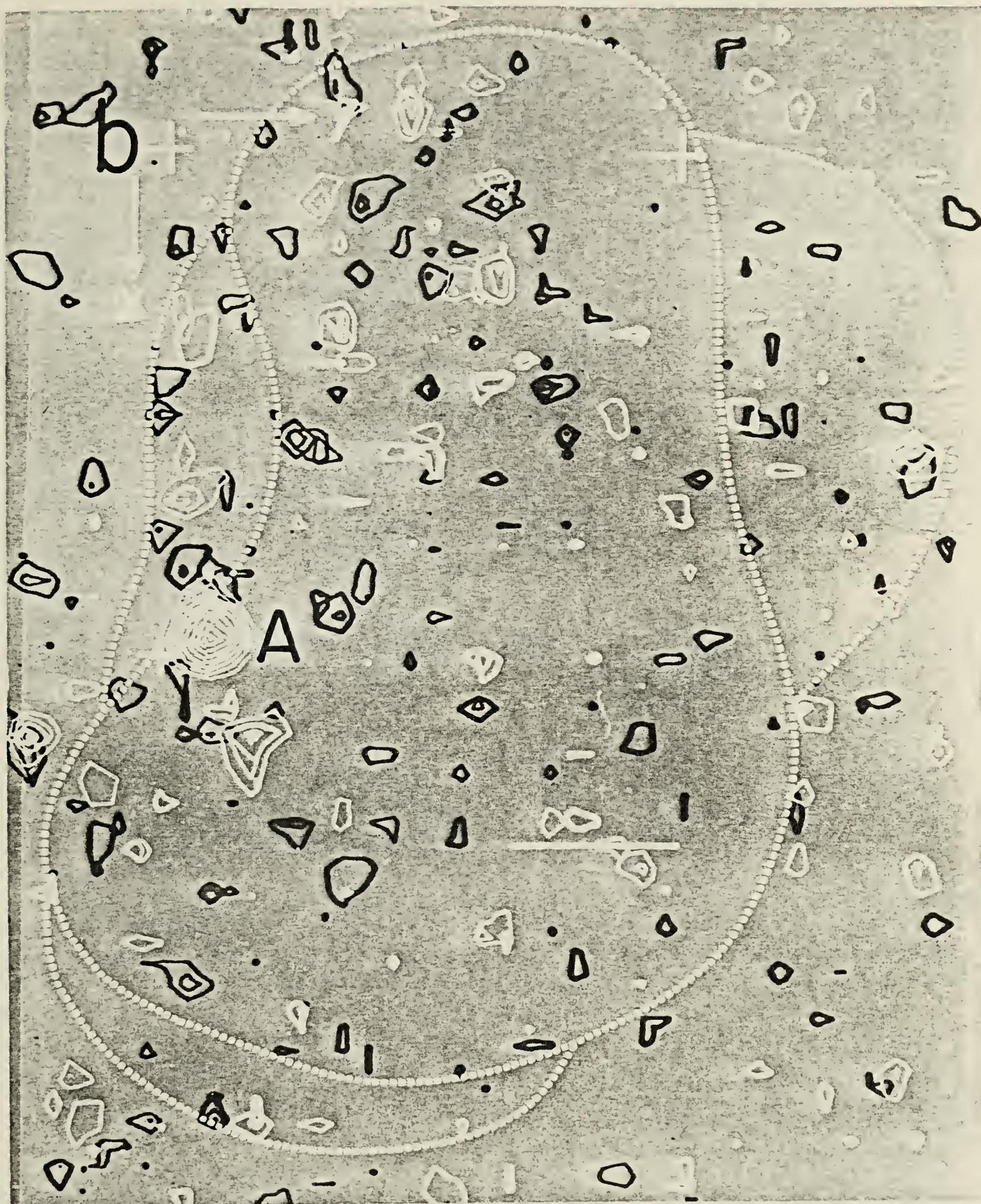


Figure 18B



Figure 18C

between the base ring of the bound nucleotides and an aromatic amino acid residue at the allosteric site, and that in the high affinity form of the enzyme for AMP, newly formed bonds between enzyme and nucleotide allow heterotropic cooperativity.

Anderson and Graves covalently modified phosphorylase b with 8-(m-(m-fluorosulfonylbenzamido)benzylthio)adenine, and postulated that a tyrosyl residue provides the site of covalent attachment (13, 14). The amino acid composition of the pentapeptide found to bind this analogue, together with the amino acid sequence of phosphorylase (60) tentatively identify the residue of interest as tyr-155. The modified enzyme exhibited 30% of maximal activity. The independence of the modified enzyme activity on the presence of AMP suggests that the covalent binding of the AMP analogue in this case is rather similar to the usual binding of AMP in native enzyme. Anderson and Graves in fact postulate, based on space-filling models of the two molecules, that the site of attachment of the analogue is similar to that with which the phosphate group interacts in the binding of AMP (14). Without the support of "direct" visualization of the binding of the AMP analogue using x-ray crystallography, one cannot confirm this postulate. Clearly the identification of an arginyl residue as the binding post for the phosphate moiety of AMP (Chapter III) indicates that the sites of attachment cannot be identical for the two cases. However, the reactivity of this tyrosyl residue near the AMP binding site suggests that it is a possible site of attack by ferrate. Other potential candidates for ferrate oxidation include tyr-74 and tyr-75, which are both situated in the anion-binding pocket.

The covalent attachment of an AMP analogue by Hulla and Fasold

involved the formation of a thioether bond between the 6-position of the purine moiety and an aliphatic sulfhydryl group of the protein (12). Examination of the 3-dimensional structure obtained crystallographically and the primary structure obtained by amino acid sequencing techniques strongly suggests that the modified sulfhydryl group at the AMP-binding site is that of cys-317, which is immediately adjacent to the arginyl residue identified by butanedione modification. Clearly, the orientation of the AMP analogue bound via its purine 6-position to cys-317 cannot be identical to that of AMP bound via its phosphate moiety to arg-318. Yet both of these modified phosphorylase b enzymes as well as that modified by Anderson and Graves exhibited an increase in the activity assayed in the absence of AMP as activator. These results imply that although occupancy at the anion-binding pocket is obligatory for activity in phosphorylase b, a rather wide range of flexibility exists for the mode of binding, at least in certain subsites.

In addition to the decrease in tyrosine content of ferrate-treated phosphorylase, the amino acid analyses also indicate the formation of approximately two cysteic acid residues per monomer. The modification of the cysteinyl residues, however, is not likely to be the primary inactivating reaction, since other work has indicated the ability to modify two fast-reacting sulfhydryl groups per monomer without loss of activity or large changes in structure (2-5). In phosphorylase a, the cysteic acid content remained unchanged whether the reaction was carried out in the absence or presence of glucose-1-P, yet the latter reaction mixture exhibited a higher enzymic activity. Similarly, in phosphorylase b, protection against inactivation by AMP

was accompanied by protection against loss of tyrosyl residues but not of cysteinyl residues (15).

Although modification of the sulfhydryl groups of cysteinyl residues is not likely to be the primary cause of inactivation, it may have been responsible for the loss of homotropic cooperativity of AMP binding in ferrate-treated phosphorylase b. Similar results were obtained by DTNB treatment of 4 sulfhydryl groups per dimer (43); modification of these sulfhydryl groups did not lead to changes in enzyme activity but did abolish homotropic interactions of AMP. It is possible that these same sulfhydryl groups were involved in the case of ferrate treatment. However, one can modify the fast-reacting sulfhydryl groups of phosphorylase b with another sulfhydryl reagent, iodoacetamide, without altering the allosteric properties of the enzyme (5). In addition, other possibilities cannot be excluded since other examples of loss of homotropic cooperativity have been observed. The homotropic interaction of AMP also could not be demonstrated in phosphorylase b partially inactivated by glutaraldehyde (7), although heterotropic interactions between substrates and AMP could still be demonstrated. The glutaraldehyde reagent modified about 10% of the lysyl residues of the enzyme without evidence of reaction with sulfhydryl groups. The glutaraldehyde-treated enzyme possessed essentially the same affinity for AMP as native enzyme, in contrast to a decreased affinity for AMP exhibited by both ferrate-treated and DTNB-treated enzymes. The mechanism for "desensitization" to AMP homotropic interactions in glutaraldehyde-treated enzyme thus appears to differ from those involving sulfhydryl groups. It is not clear whether the basis for ferrate-induced

desensitization is similar to either of these other cases. One also cannot dismiss the possibility for loss of homotropic cooperativity simply resulting from a population of dimers with one modified monomer no longer able to bind AMP and one unmodified monomer which does still bind AMP.

These data suggest that modification of one or more tyrosyl residues is the inactivating reaction, but it is possible that amino acid residues other than tyrosine and cysteine may have been modified. No independent assay was carried out to determine the cysteine content, but the formation of cysteic acid residues noted in our analyses is consistent with the results of Lee and Benisek on phosphorylase b (15). Small changes in the content of especially the more abundant residues such as arginine, aspartic acid and glutamic acid, could well go undetected by amino acid analysis techniques. However, these residues are not typically susceptible to oxidation. No attempts were made to determine tryptophan content in this study, since Lee and Benisek (15) had found little change in the tryptophan content of ferrate-treated phosphorylase b.

The inability to visualize by x-ray crystallography at 4.5 Å^o resolution the particular group or groups which have been chemically modified is not surprising since the reaction with ferrate may only lead to a minor alteration in the residue, perhaps a simple oxidation of a phenolic hydroxyl group to a carbonyl group. Nevertheless, the evidence suggests modification of one or more tyrosyl groups near the AMP-binding site; alternatively, tyrosyl residues may be modified and affect AMP binding by indirectly conveying conformational changes.

The decrease in occupancy of AMP at the anion-binding site in modified crystals of phosphorylase a and the decrease in homotropic cooperativity for AMP binding manifested by phosphorylase b in solution both provide evidence for a change in the mode of or a decrease in the extent of binding of AMP.

Two lines of evidence suggest that ferrate treatment enhances the affinity of binding of ligands at the glucose-binding locus. Firstly, the crystallographic work shows that the binding of glucose-1-P to the glucose-binding locus is favored in the ferrate-treated enzyme. Secondly, there is a slight enhancement of glucose inhibition, as indicated by a decreased (+ glucose/- glucose) activity ratio. If, as suggested by the work with butanedione (Chapter III), the glucose-binding locus is the active site, why then does ferrate treatment inactivate the enzyme? It is possible that in the native enzyme, the binding of AMP (or of glucose-1-P) at the anion-binding site may trigger a change in the tyrosyl residues perhaps in their interaction with ligands or with other residues, which then in turn releases a restriction at the glucose-binding locus. In the ferrate-treated enzyme, oxidation of the tyrosyl residues may also release this restriction but may alter the mode of binding at the glucose-binding locus such that greater inhibition is exerted by glucose, while catalysis of substrates is hindered. Conversely, the protection effects of ligands might be explained by a reciprocal action of the ligands transmitted to the tyrosyl residue via conformational changes which render the residue less susceptible to reaction. The protection against inactivation afforded by AMP, AMP analogues and acetate in phosphorylase b (15) contrasts with the lack of this pro-

tection in phosphorylase a (Table 5). These results imply that phosphorylases a and b differ in their relationship between the modified tyrosyl residue(s) and binding of ligands at the anion-binding site, and that the conformational changes induced on phosphorylation of ser-14 in the former case differ from those brought about by binding of AMP in the latter case, although both allow manifestation of enzymic activity. These trends echo those previously observed for ligand protection against butanedione inactivation in solution as well as ligand binding in butanedione-treated crystals (Chapter III).

Modification of Carboxyl Groups with Carbodiimide

A. Introduction

The importance of a group with a pK_a of approximately 6 indicated by pH kinetics (8, 44) suggests the possible role of a carboxyl group in the catalytic mechanism of phosphorylase. The involvement of carboxyl groups could conceivably be analogous to that in lysozyme, in which a carboxyl group in a hydrophobic environment transfers its proton to the glycosidic oxygen to create a carbonium ion intermediate, which is then stabilized by the ionized carboxyl group of a second acidic residue. This hypothesis is consistent with the specific inhibition of phosphorylase b by 1,5-gluconolactone, a potential analogue for a carbonium ion intermediate. The inhibition is competitive with glucose-1-P, but not with either glycogen or with arsenate (45). These results were interpreted to mean that 1,5-gluconolactone competes for the glucosyl transfer site, and that in a transition state enzyme-glucosyl complex, the transfer site is occupied by the glucosyl residue in a half-chair conformation.

The reactions of 5-diazo-1-H-tetrazole (DHT) and of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate in the presence of glycine ethyl ester both suggest tentative evidence for an essential carboxyl group in phosphorylase (46). In either case, complete inactivation resulted from the incorporation of one mole of reagent per mole of monomer. However, DHT is a relatively non-specific reagent for

several amino acid residues, and the identification of the modified functional group as a carboxyl group is only speculative. Although comparison of the absorption spectra of model compounds with those of DHT-treated enzyme indicated that the modified residue was not likely to be tyrosine, histidine, lysine or tryptophan, no corresponding data could be obtained for arginine, serine or threonine (47). Protection studies with substrates did not give conclusive evidence as to whether or not modification occurred at the active site.

The work with carbodiimide in the presence of glycine ethyl ester is more definitive. The studies of Hoare and Koshland (48) provide good evidence of the specificity of water-soluble carbodiimides in the presence of various nucleophiles for carboxyl groups. Although carbodiimide may potentially react with the active hydrogens in both cysteinyl and tyrosyl residues, the inactivation of phosphorylase is not likely due to these side reactions since inactivation is neither affected by the presence of mercaptoethanol nor reversed by the addition of hydroxylamine (49). Avramovic-Zikic et al (46) showed that the incorporation of 1 to 1.5 moles of ^{14}C -glycine ethyl ester per monomer resulted in complete inactivation, and subsequently isolated radioactive dipeptides identified as γ -glutamyl glycine and β -aspartyl glycine. However, these workers were unable to isolate unique fragments bearing the modified residues.

These studies prompted further investigation of the modification of carboxyl groups in microcrystals and crystals of phosphorylase a in an attempt to identify the site(s) of modification and to substantiate the hypothesis for the involvement of carboxyl groups in the catalytic mechanism.

B. Results

1. Phosphorylase a in Solution

The reaction of phosphorylase a in solution with 10 mM carbodiimide and/or 50 mM glycine ethyl ester at pH 6.7 is shown in Figure 19. In the absence of glycine ethyl ester, there was an initial rapid inactivation until 70% activity remained, at which point little further change occurred. These results may be explained by an initial build-up of labile O-acylisourea derivative which in the absence of any nucleophilic species or base may simply be hydrolyzed in aqueous solution to regenerate the original carboxyl group, or else may undergo a slow isomerization to the more stable N-acylurea (see scheme in Figure 20). The concentrations of the O-acylisourea and N-acylurea species, which are manifested by the decrease in enzymic activity, reach a maximum when the second-order rate of formation is equaled by the pseudo-first order rate of disappearance by hydrolysis. The addition of a nucleophile to the O-acylisourea intermediate produces a stable carboxylate derivative and releases the isourea, thereby shifting the equilibrium towards complete reaction of carboxyl groups. The curves in Figure 19 corresponding to the reaction in the presence of the nucleophile glycine ethyl ester indicate the enhancement of inactivation by the nucleophile by the formation of a stable carboxylate-glycinamide derivative.

The slight but significant drop in activity of enzyme treated only with glycine ethyl ester was rather unexpected. It is possible that nucleophilic attack on some residue affected the affinity for substrates or restricted conformational mobility. It is unlikely that

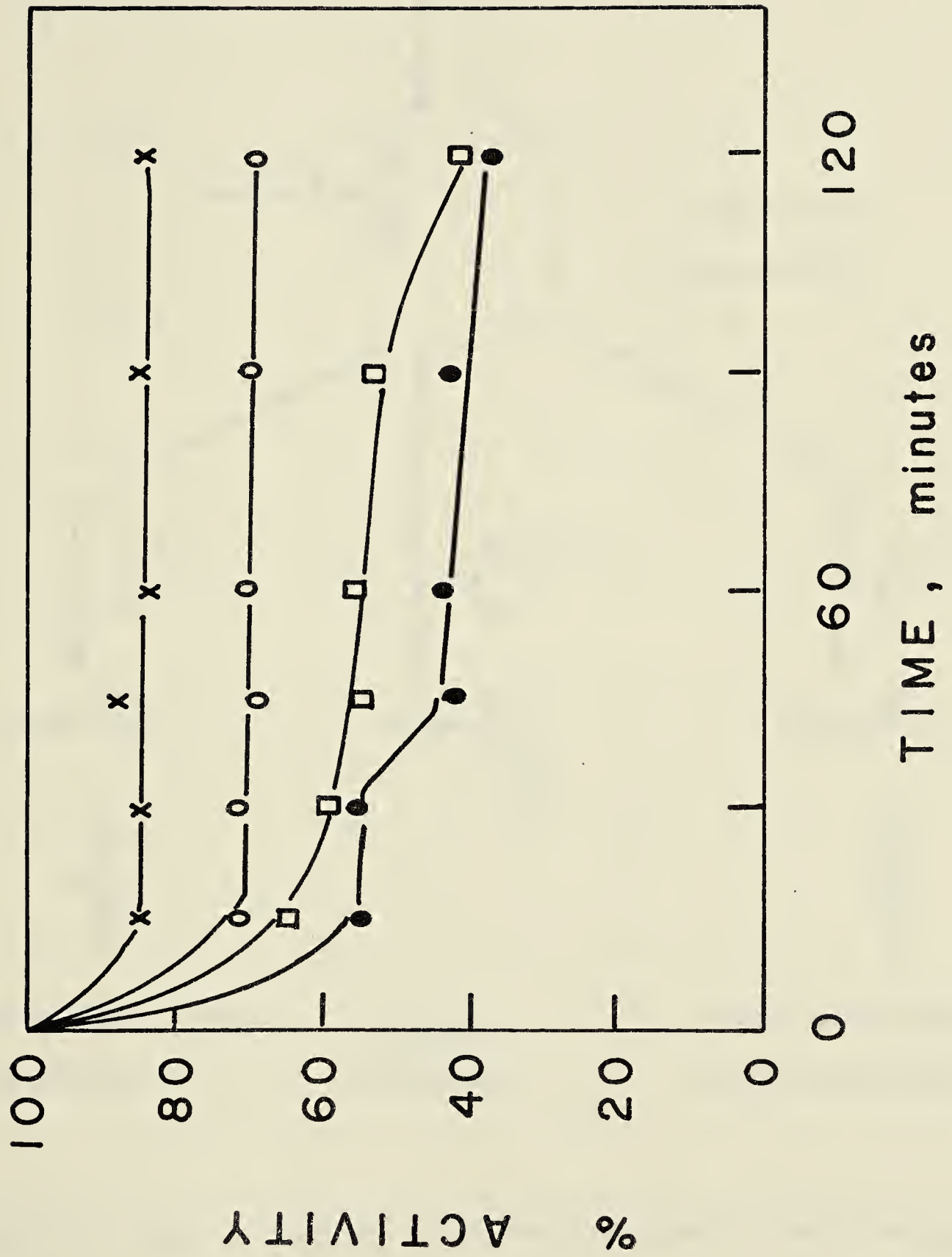


Figure 19.

Inactivation of phosphorylase a in solution at pH 6.7
by carbodiimide in the presence or absence of nucleophile.

The reaction conditions were as follows:

- X 1 mg/ml enzyme, 50 mM glycine ethyl ester
- 0 1 mg/ml enzyme, 10 mM carbodiimide
- 1 mg/ml enzyme, 10 mM carbodiimide, 50 mM
glycine ethyl ester
- 8 mg/ml enzyme, 10 mM carbodiimide, 50 mM
glycine ethyl ester



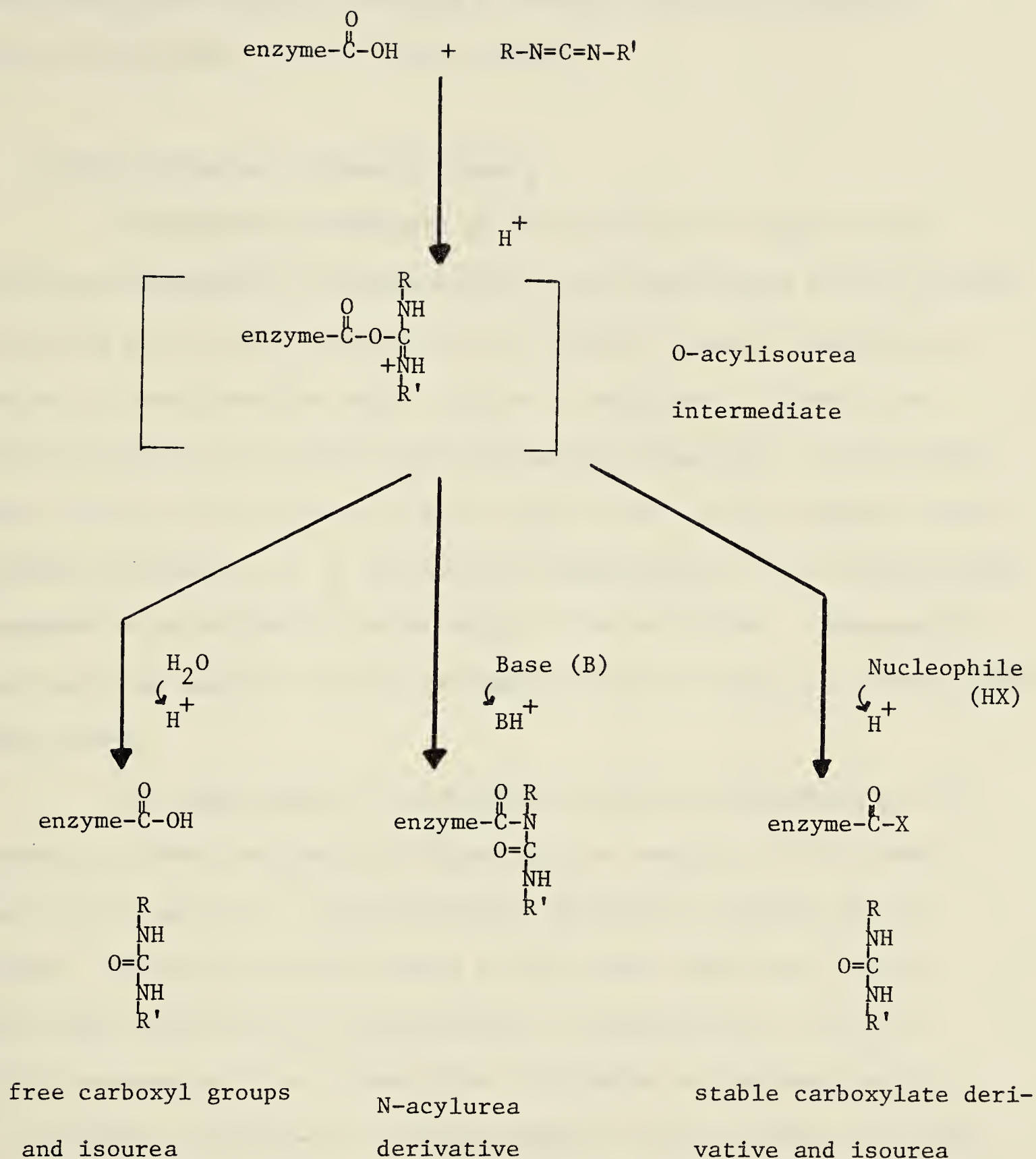


Figure 20. Scheme of carboxyl group modification by a water-soluble carbodiimide in the absence and presence of a nucleophile (adapted from Perfetti et al (51)).

the modification directly affected a residue involved in catalysis since considerable activity still remained.

2. In Microcrystals of Phosphorylase a

Preliminary experiments of the reaction of 1 mg/ml cross-linked microcrystals of phosphorylase a with carbodiimide in the presence of 100 mM glycine ethyl ester indicated that the rate of inactivation was much slower than the rates observed in solution. Although the inactivation was accelerated upon increasing carbodiimide concentration over the range from 5 mM to 20 mM, an upper limit to the reagent concentration was imposed due to the extensive aggregation of the microcrystals observed at carbodiimide concentrations of 40 and 50 mM. Subsequently, the reactions employed 20 mM carbodiimide in the presence of 100 mM glycine ethyl ester.

The enhancement of inactivation rate by carbodiimide in the presence of added nucleophile (Figure 21) was similar to the trends observed in solution. The inactivation appeared to consist of two phases. The initial phase leading to 30% inactivation was followed by a short lag prior to a second phase of inactivation. The "lag" period observed at 3 or 4 hours after initiation of the reaction is a reproducible phenomenon. This may suggest that two groups are being modified, a lag period elapsing during which conformational changes resulting from modification of the first group are prerequisite for susceptibility of the second group to reaction.

The effects of the allosteric activator AMP and the competitive inhibitor UDP-glucose, alone or in combination, are seen in Figure 22.



Figure 21.

Inactivation of 1 mg/ml phosphorylase a micro-crystals at pH 6.7 by carbodiimide in the absence and presence of nucleophile.

The reaction conditions were as follows:

- 100 mM glycine ethyl ester
- 20 mM carbodiimide
- X 20 mM carbodiimide and 100 mM glycine ethyl ester

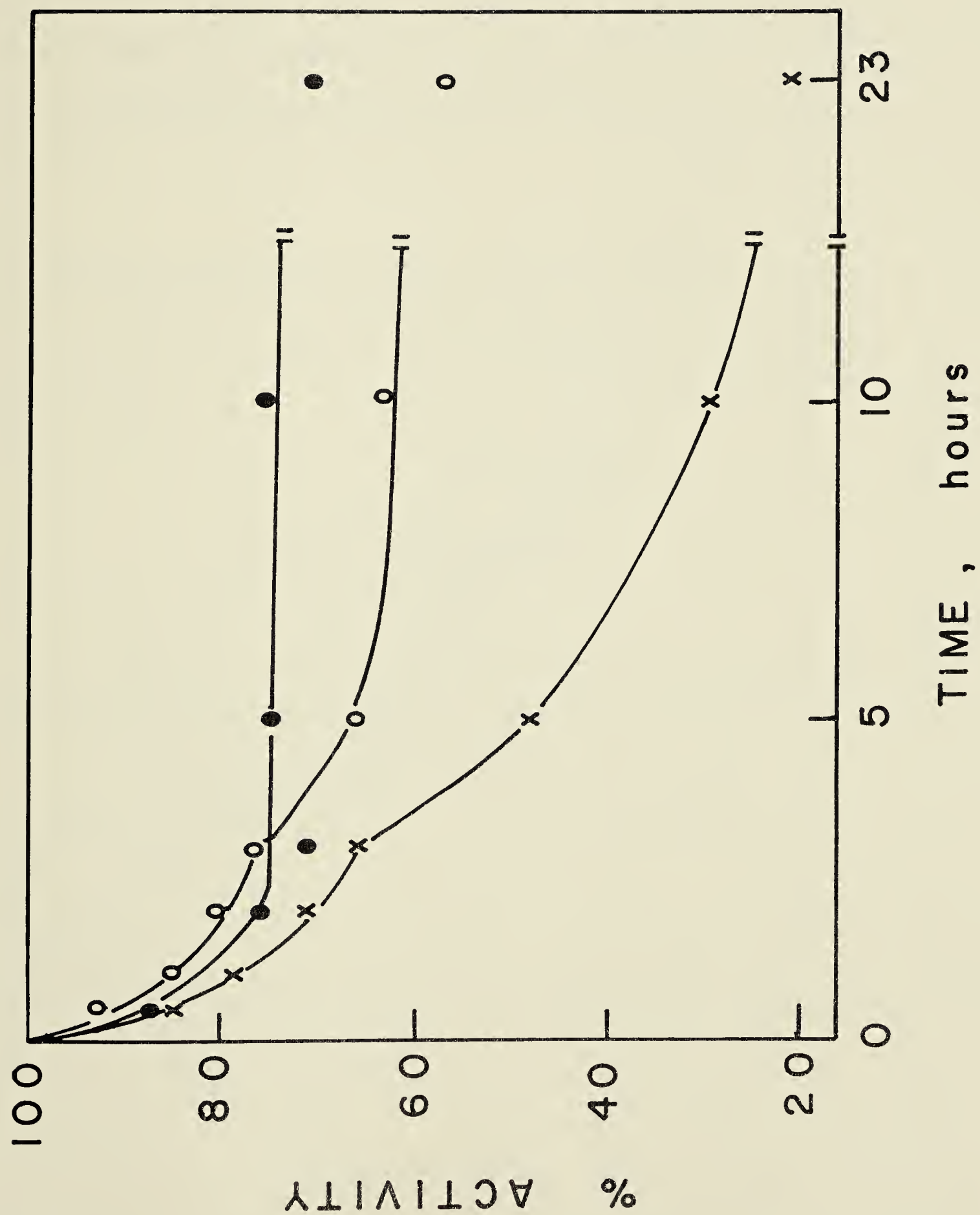


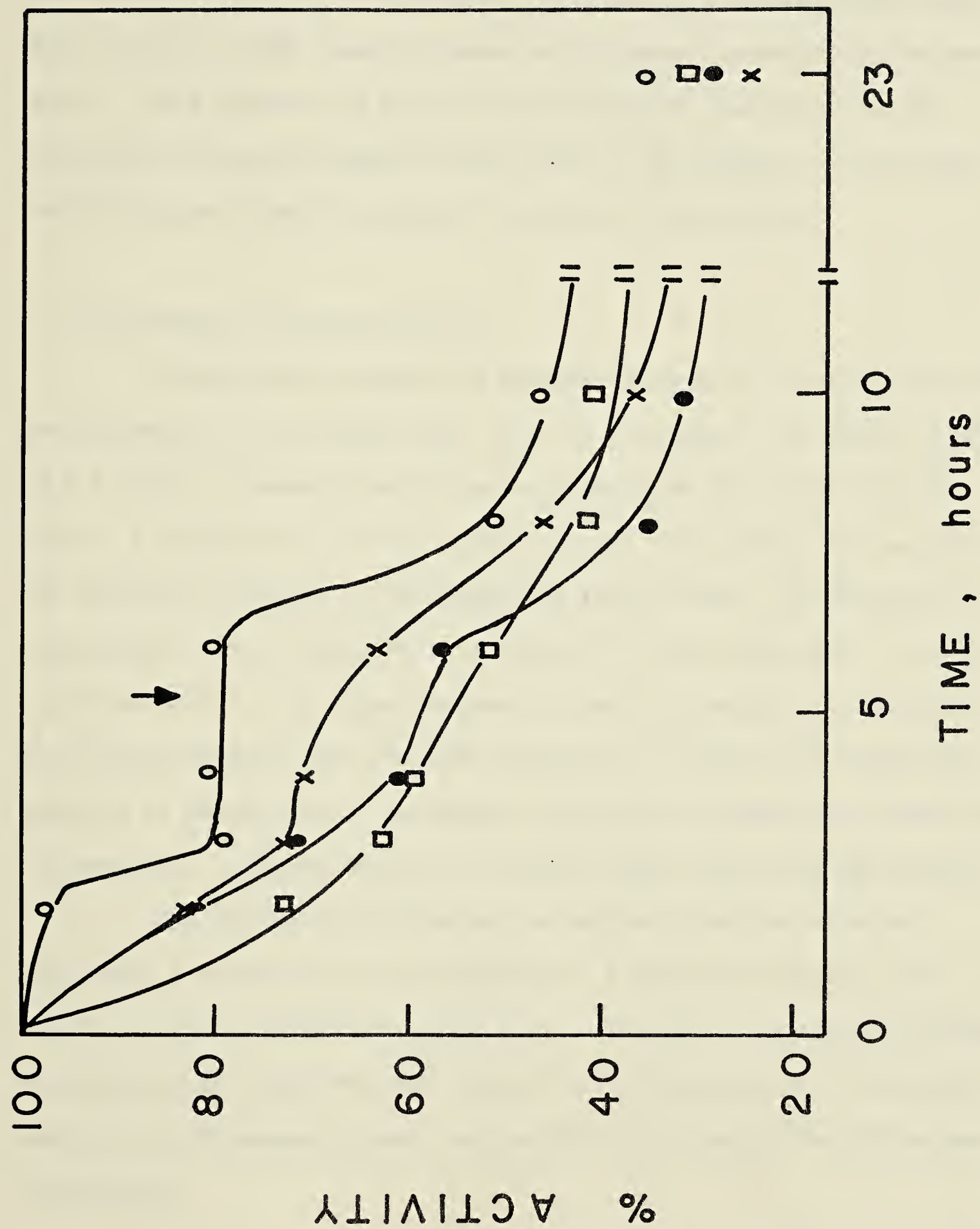
Figure 22.

Effect of ligands on the inactivation of phosphorylase a microcrystals by carbodiimide in the presence of nucleophile.

Reaction mixtures contained 1 mg/ml phosphorylase a, 20 mM carbodiimide and 100 mM glycine ethyl ester at pH 6.6, and the following ligands:

X	none
●	1 mM AMP
0	15 mM UDP-glucose
□	1 mM AMP and 15 mM UDP-glucose

The reaction media were replaced with fresh solutions at the time indicated by the arrow, by spinning down the microcrystals in a clinical centrifuge and resuspending them in freshly prepared solutions.



For this experiment, in all cases, after 5 1/4 hours, the microcrystals were spun down at low speeds and resuspended in fresh reagent and buffer solutions in order to avoid possible non-specific reactions with breakdown products of the reagents formed on prolonged incubation in aqueous media. AMP increased the rate of inactivation of the enzyme, while UDP-glucose protected against inactivation. The presence of both AMP and UDP-glucose further increased the rate of inactivation.

3. In Crystals of Phosphorylase a

Cross-linked crystals of phosphorylase a were reacted with 20 mM carbodiimide-100 mM glycine ethyl ester in 5 mM BES-0.5 mM EDTA-1.5 mM DTT pH 6.7 buffer. Reagents and buffer were replaced with fresh solutions every 3 1/2 hours for the first twelve hours, after which the reaction was allowed to proceed for an additional twelve hours. Termination of the reaction was accomplished by washing with fresh 10 mM BES-1 mM EDTA buffer at pH 6.7. The large decrease in ionic strength in transferring the reacted crystals from reaction conditions to fresh buffer may have resulted in osmotic shock, leading to formation of superficial cracks in the crystals. However, within 15 minutes, these cracks had disappeared.

The difference Fourier map calculated using the structure amplitudes F (carbodiimide, nucleophile) - F (native) indicated that fairly extensive changes had taken place. The root mean square difference of the structure amplitudes was about 15 %. Various peaks of electron density of approximately equal peak heights were seen on the difference Fourier map.

Milder reaction conditions were used in a second trial with

Figure 23.

Difference Fourier electron density map at 4.5 \AA resolution using the structure amplitudes $F(\text{carbodiimide}) - F(\text{native})$, of a crystal of phosphorylase a reacted with 2 mM carbodiimide-10 mM glycine ethyl ester at pH 6.7.

Positive changes in electron density are indicated by white contour lines, negative changes by dark lines. Dotted lines delineate the molecular outlines at Z coordinates of 0.40 to 0.46 and 0.22 to 0.27, which include the anion-binding and glucose-binding sites, respectively. X and Y coordinates at 0 and 0.25 are indicated by +. The horizontal bar represents 10 \AA .

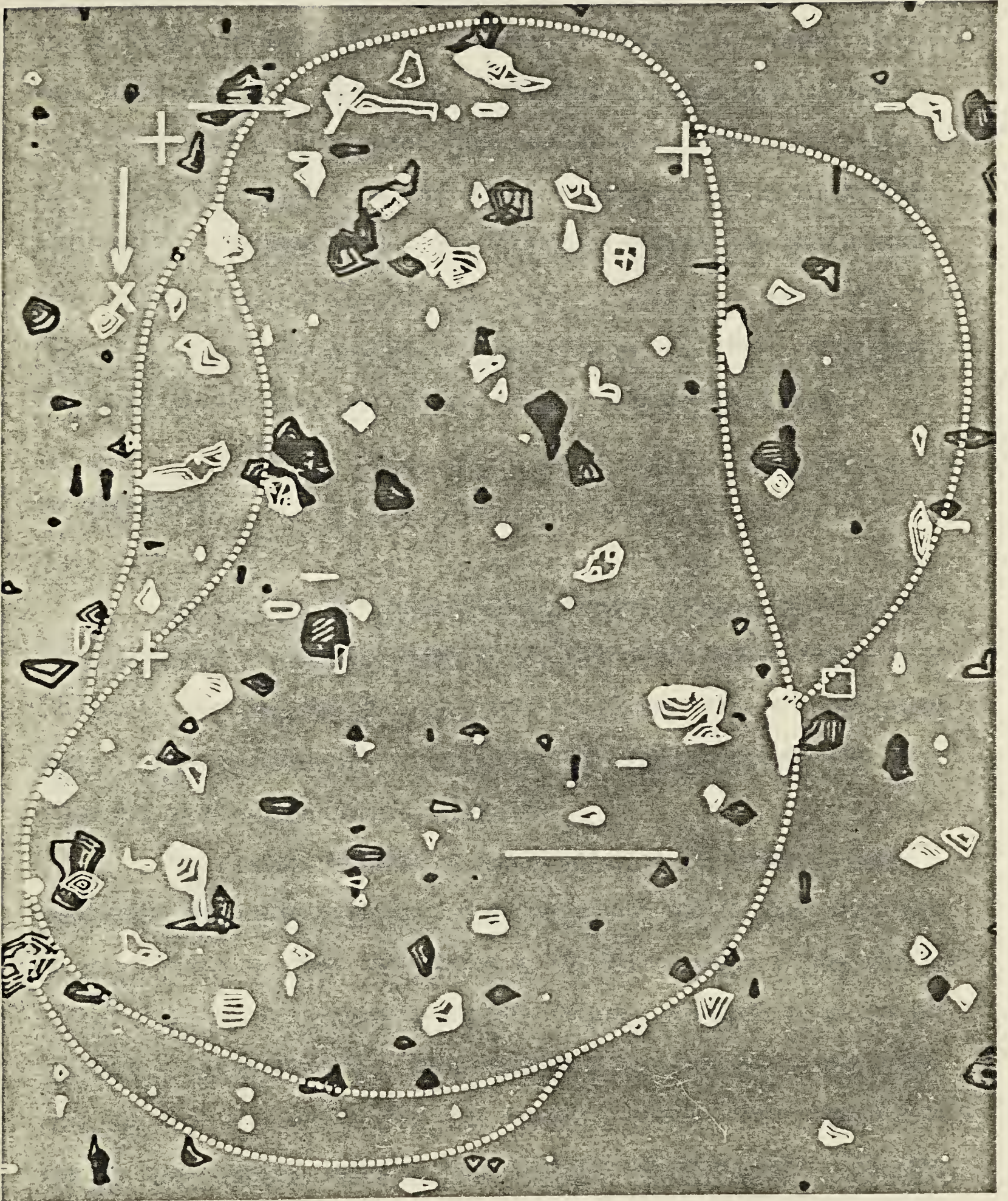


Figure 23

both native and cross-linked crystals. The reagents, 2 mM carbodiimide and 10 mM glycine ethyl ester, were replaced with fresh reagent solutions once at 4 hours after initiation of reaction, and the reaction was terminated at 16 hours by exhaustive washing with buffer. The difference Fourier map calculated using the structure amplitudes F (carbodiimide, nucleophile) - F (native) at 4.5 Å resolution (Figure 23) showed again many peaks of electron density. No clear conclusions could be made since none of the peaks were particularly intense, nor did these correspond to the known binding sites for ligands noted in the native enzyme.

C. Discussion

The results of inactivation of phosphorylase a in the microcrystalline and crystalline forms of the enzyme are rather difficult to interpret. Although the general trends for inactivation are similar to those in solution observed by Avramovic-Zikic and colleagues (46, 49), there are also some distinct differences.

The complex kinetics of inactivation in the microcrystals suggesting sequential modification of two groups may in fact exist also in solution but may have been masked by the faster rates of inactivation and greater ease of conformational flexibility. Although a lag was not apparent in previous work on phosphorylase a in solution, it was noted that the loss of activity with time was not a simple first-order function but was more complex, being at least biphasic in nature (49). The restrictions imposed by crystallization and cross-linking procedures may have decreased the reactivity of the second group of carboxyl groups in

the microcrystals, and thus necessitated the prior reaction of one group before the second becomes reactive.

In both solution and microcrystals, the rate of inactivation was greater in the presence of AMP or of AMP and UDP-glucose. This implies that the carboxyl groups become more susceptible to modification as a result of the changes induced upon binding at the anion-binding site. The presence of the competitive inhibitor UDP-glucose alone served to retard the rate of inactivation in the microcrystals and is suggestive of direct protection of a carboxyl group at the active site. Decrease in the rate of inactivation was also observed for phosphorylase a in solution in the presence of glucose, another competitive inhibitor of glucose-1-P (50). However, the effects of substrate on inactivation of the enzyme in solution are complicated by the accompanying effects on dimer-tetramer interconversions (49, 50).

The multiplicity of sites of reaction implied in the difference Fourier maps was not totally unexpected, in the light of the vast number of potentially reactive carboxyl groups - 114 per monomer (36). Although studies employing ^{14}C -glycine ethyl ester had indicated complete inactivation of the enzyme in solution after a stoichiometric incorporation of reagent, the radioactivity was shared among γ -glutamylglycine and β -aspartylglycine dipeptides. The highest radioactivity, 70% of the total incorporation, was found in the region corresponding to a standard of γ -glutamylglycine while another 30% of the radioactivity was found in a region corresponding to β -aspartylglycine. Further experiments failed to prove that the radioactivity was confined to unique protein sequences, and the x-ray crystallographic evidence in this study would suggest that

in fact several carboxyl groups had been modified.

The apparent discrepancy between the non-specificity observed by crystallography and the specificity implied by the 1:1 stoichiometry between enzyme inactivation and reagent incorporation may perhaps in part be explained by the difference in conditions for reaction - pH 6.7 for crystals of phosphorylase a in the former case, and pH 5.2 for phosphorylase b in solution in the latter case. The rate of inactivation of phosphorylase in solution was found to increase with decreasing pH (49), suggesting that the rate-limiting step is the protonation of a nitrogen on the carbodiimide. However, the subsequent attack of a carboxyl group on the carbodiimide is dependent on the ionized form of the carboxyl group. At higher pH, a greater proportion of the carboxyl groups are ionized and become reactive. Avramovic-Zikic et al (49) also had found some differences between phosphorylases a and b in solution in their reaction with carbodiimide. The reaction with phosphorylase a was independent of the presence of nucleophile, exhibited complex kinetics of inactivation and a smaller dependence on pH.

One can only conclude that carbodiimide in the presence of nucleophile inactivates phosphorylase. The site of the inactivating reaction remains unknown.

Chapter VI

Modification of Histidyl Residues with Diethyl Pyrocarbonate

A. Introduction

The imidazolyl group of a histidyl residue is a likely candidate for the group responsible for a pK near 7 in the alkaline limb of V_{\max} -pH profiles (8). Preliminary work in this laboratory (9) on the photooxidation of phosphorylase b in the presence of methylene blue indicated that inactivation was accompanied by a loss of histidyl residues. However, studies on pH dependence of the inactivation were inconsistent with the destruction of histidyl residues as being the cause of inactivation. No particular histidyl residue could be singled out as an essential one, modification of over one-third of the histidyl residues accompanying 70% inactivation. Sedimentation velocity studies indicated some dissociation of the dimeric form of the enzyme into monomers. The substrates, glucose-1-P and glycogen, and the allosteric activator AMP each increased the rate of inactivation, but the effect was most pronounced when both AMP and glucose-1-P were present together.

Rather similar results were observed by Kamogawa and Fukui (11) using photooxidation in the presence of rose bengal. Complete inactivation was accompanied by destruction of 4 to 5 histidyl residues, and the rate of inactivation increased with pH. Gross changes of conformational structure were indicated by the tendency to form aggregates and by a partial release of pyridoxal-5'-phosphate.

The increase in rate of inactivation by AMP followed a sigmoidal curve, the effect being saturated at an AMP concentration of 0.5 mM. The rate was decreased by ATP, ADP, IMP and glucose-6-P and not affected by the addition of substrates.

These studies, while strongly suggestive of essential histidyl residues, were inconclusive due to the large number of modified histidyl residues, the effects on subunit structure and inherent nonspecificity of photooxidation. Recently, identification of histidyl residues at active sites has been facilitated through the use of the reagent diethyl pyrocarbonate, also named ethoxyformic anhydride. Diethyl pyrocarbonate has been widely used in nucleic acid biochemistry; it reacts with all major RNA bases except guanine as well as with a wide variety of ribonucleosides and ribonucleotides (52). More recently, it has also been recognized as being relatively specific for histidyl residues at a pH near 6 and low reagent concentrations. Although model studies have shown reaction with side chains of histidine, lysine, tyrosine, cysteine, serine and arginine residues, a survey of a range of proteins indicates that at pH values lower than 7, generally only histidyl residues and sometimes amino groups are modified (53). The modification of amino groups as the inactivating reaction may be eliminated if the inactivation can be reversed in the presence of hydroxylamine at neutral pH (54). Formation of carbethoxyhistidine is readily identified by its strong absorbance at 240 nm, with $\epsilon = 3600 \text{ M}^{-1} \text{ cm}^{-1}$ (26). The destruction of one or more essential histidyl residues as the cause of inactivation is also often indicated by a characteristic pH dependence of inactivation, since the unprotonated

histidyl residue is proposed to be the reactive species (26).

B. Results

1. Phosphorylase b

Figure 24 illustrates the reaction of phosphorylase b with varying concentrations of DEPC at pH 6.0. Complete inactivation resulted within five minutes of treatment with 7.2 mM DEPC, but in order to encourage greater specificity of reaction and to facilitate characterization of the kinetics of reaction, lower reagent concentrations and longer incubation periods were chosen for subsequent studies.

The ultraviolet difference spectra of DEPC-treated against native phosphorylase b are shown in Figure 25. The spectra show a peak at about 238 nm, which is characteristic of the formation of carbethoxyhistidyl residues. The lower curve represents the difference spectrum taken shortly after initiation of the reaction, the enzyme being approximately 45% inactivated. The change in absorbance at 240 nm represents the formation of between 5 to 6 carbethoxyhistidyl residues. The lack of a change in absorbance at 278 nm confirms the lack of tyrosine modification. O-Ethoxyformylation of the model compound N-acetyl-L-tyrosine ethyl ester has been reported to show a difference spectrum with a minimum at 278 nm, with $\Delta\epsilon=1310 \text{ M}^{-1} \text{ cm}^{-1}$ (55). Only after two hours of DEPC treatment (upper curve) did a slight dip in the difference spectrum at 278 nm occur, corresponding to the modification of about 2 or 3 out of a total of 34 tyrosyl residues per monomer. At this stage, approximately 90% of the 19 histidyl residues per monomer had been modified.

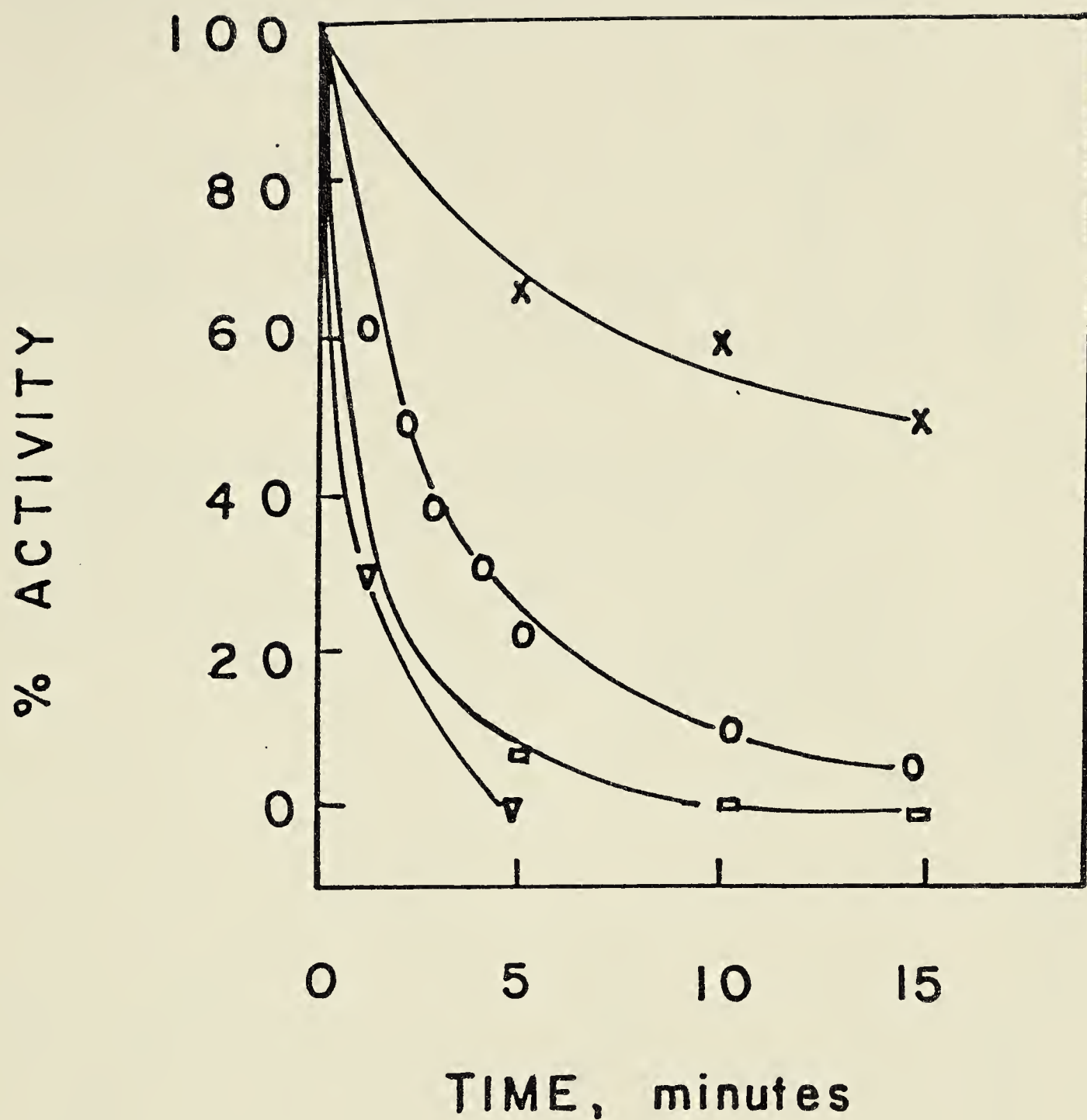


Figure 24.

Time course of inactivation of phosphorylase b as a function of varying DEPC concentrations at pH 6.0.

Phosphorylase b (0.5 mg/ml) in 50 mM glycerophosphate-1 mM EDTA buffer at pH 6.0 was reacted with 0.46 mM (X), 2.3 mM (O), 4.6 mM (□) and 7.2 mM (▽) DEPC.

Figure 25.

Ultraviolet difference spectra of DEPC-modified
against native phosphorylase b (5.1 μ M).

The lower curve represents the difference spectrum taken
1 to 2 minutes after initiation of the reaction with 2.3 mM
DEPC at pH 6.0. The upper curve represents the difference
spectrum after 2 hours of reaction time.

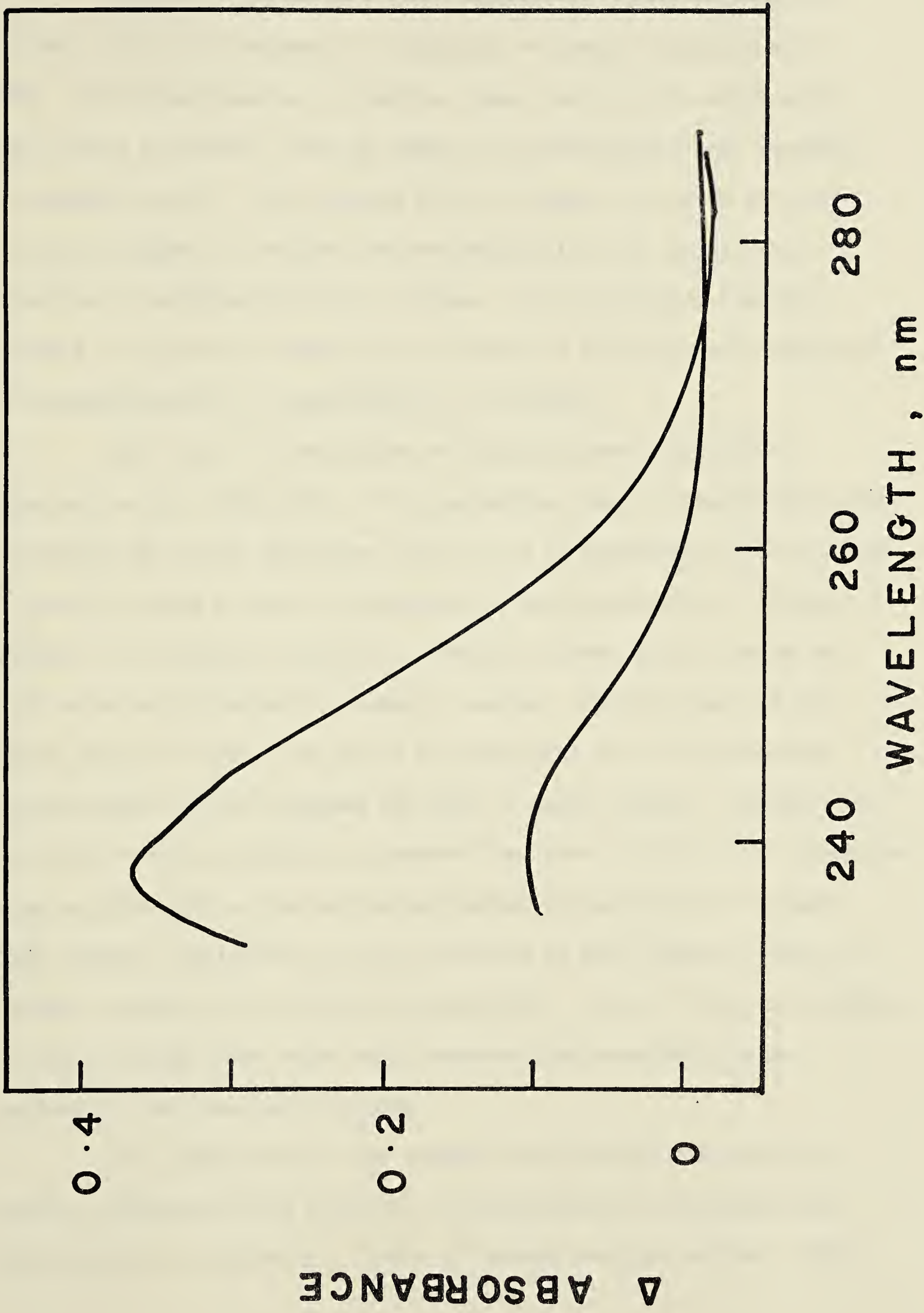


Figure 26 illustrates the formation of carbethoxyhistidyl residues formed per monomer as a function of time of reaction with DEPC. At fifteen minutes of reaction time, the rate of modification had started to plateau, and the number of carbethoxyhistidyl residues per monomer was 15. The combined data of Figures 24 and 26 are re-plotted in Figure 27 to show the concomitant loss of activity and formation of modified histidyl residues. No particularly reactive residues are apparent, complete loss of activity having been accompanied by the modification of approximately 15 residues.

The rate of inactivation of phosphorylase b by DEPC is dependent on pH (Figure 28). The increasing rate of inactivation with increasing pH in the range from 5.25 to 6.8 is consistent with the loss of activity being a direct consequence of the destruction of histidyl residues. No rigorous analysis to interpret these data in terms of a pK value was attempted for several reasons. Firstly, data at pH values above 7.0 would not likely be meaningful due to the enhanced reaction with tyrosyl residues and lysyl ϵ -amino groups. Secondly, the inactivation did not adhere to pseudo-first order kinetics, the deviation being attributable either to the hydrolytic decomposition of reagent under aqueous conditions or to the reaction of more than one group of histidyl residues with different reactivities. Thus it was not possible to obtain pseudo-first order rate constants and detailed kinetic analysis of the data was precluded.

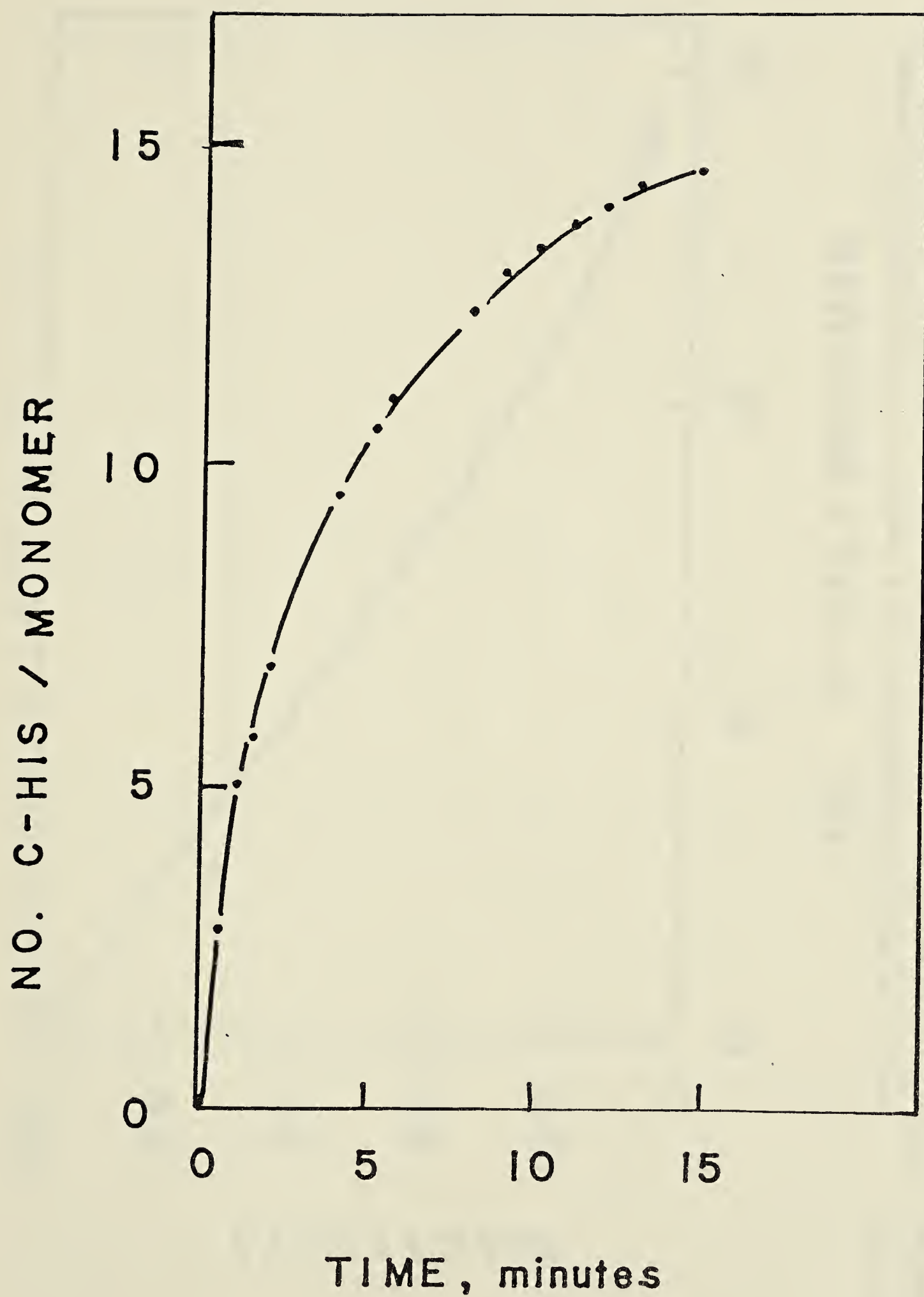
The selectivity of the reagent for histidyl residues was further confirmed by the reversal of inactivation by treatment with hydroxylamine at neutral pH. Table 10 summarizes the results. Quite

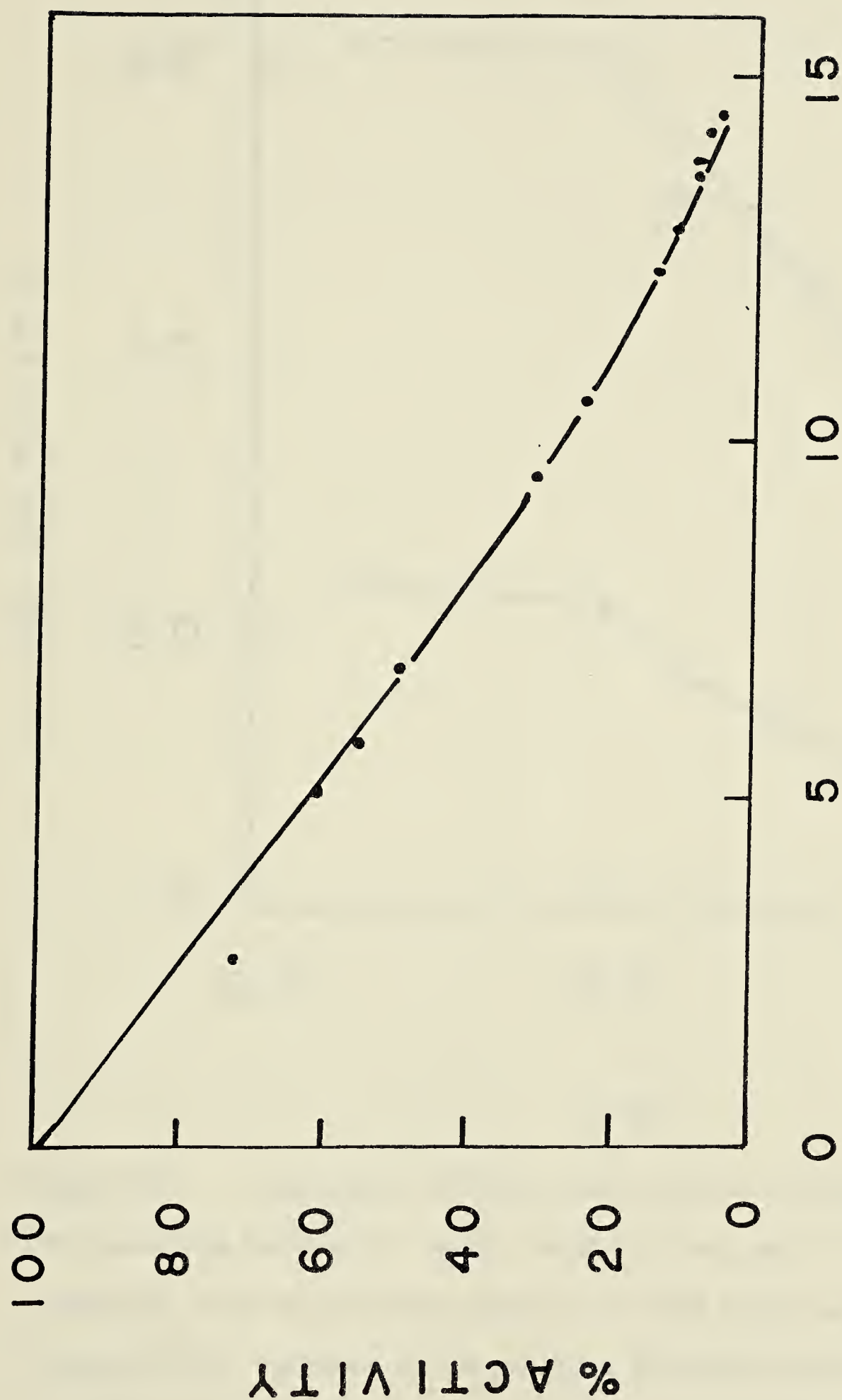


Figure 26.

Formation of carbethoxyhistidyl residues as a function of time of reaction of 0.5 mg/ml phosphorylase b with 2.3 mM DEPC at pH 6.0.

The number of carbethoxyhistidyl residues per monomer of phosphorylase b (C-His/monomer b) was calculated from the change in absorbance at 240 nm, using $\Delta\epsilon_{240} = 3600 \text{ M}^{-1} \text{ cm}^{-1}$.





NO. C-HIS / MONOMER

Figure 27. Loss of activity of phosphorylase \underline{b} as a function of carboxyhistidyl residue formation. The data in Figures 24 and 26 were combined to obtain this plot for the reaction of 0.5 mg/ml phosphorylase \underline{b} with 2.3 mM DEPC at pH 6.0.

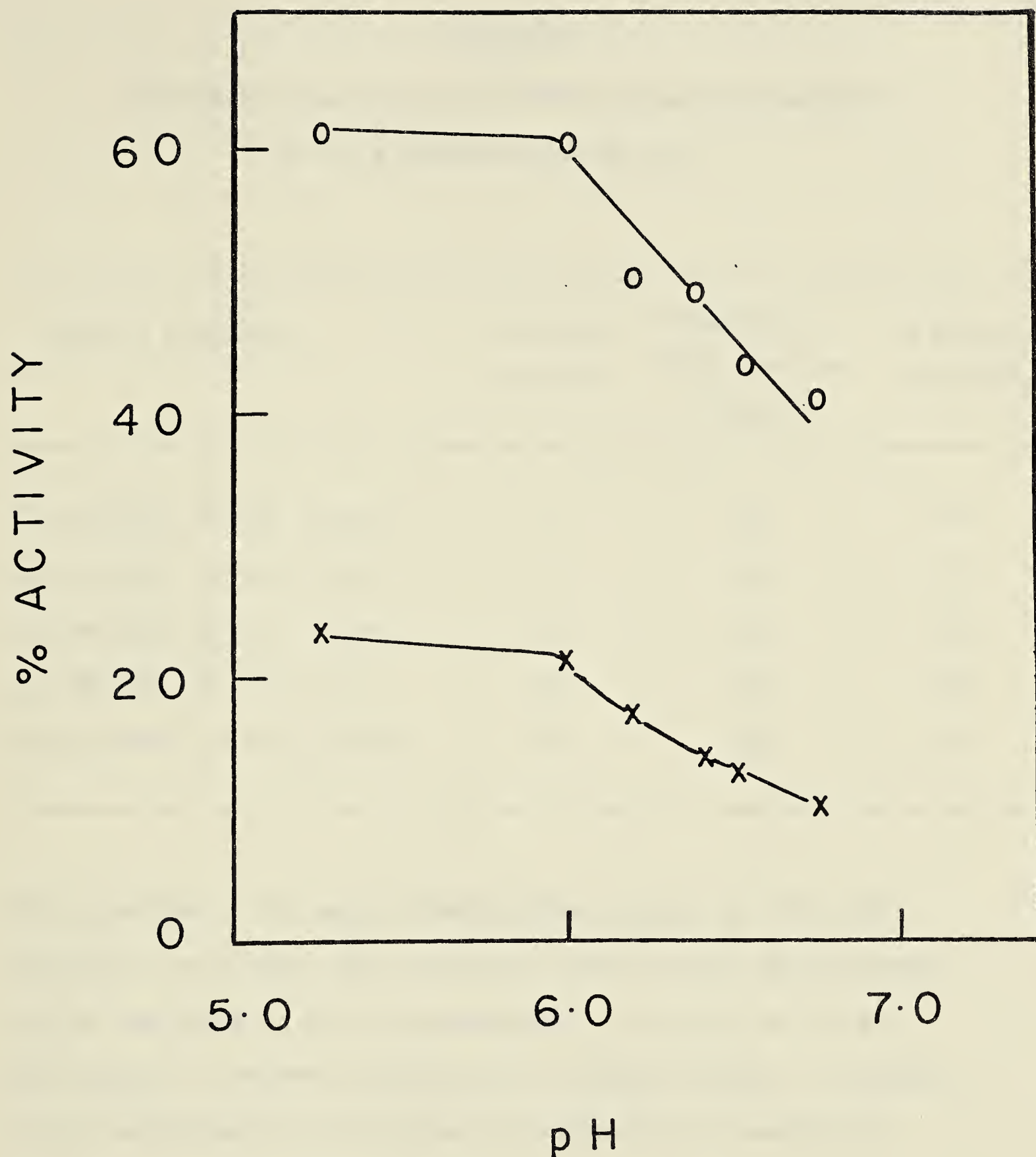


Figure 28. Dependence of DEPC inactivation of phosphorylase b on pH. The reactions between 0.5 mg/ml phosphorylase b and 2.3 mM DEPC were performed in 50 mM glycerophosphate-1 mM EDTA buffer adjusted to the indicated pH. Aliquots of the reaction mixtures were assayed for activity at pH 6.8 after 1 minute (O) and 5 minutes (X) reaction time.

Table 10

Reversal of inactivation of DEPC-treated phosphorylase b
by 0.5 M hydroxylamine at pH 7

Reaction Conditions	% Activity initially	Time after NH_2OH addition, minutes	% Activity after NH_2OH
1.8 mM DEPC, pH 6.8, 10 min	4	60	36
1.8 mM DEPC, pH 6.0, 10 min	6	90	56
2.3 mM DEPC, pH 6.0, 10 min	5	30	51
2.3 mM DEPC, pH 6.0, 5 min	21	60	80
0.57 mM DEPC, pH 6.0, 10 min	46	20	85

After reaction of 0.5 mg/ml phosphorylase b under the indicated conditions, an aliquot was removed for assay and then hydroxylamine (NH_2OH) was added to 0.5 M concentration. 1.2 or 2.5 mM DTT was also present to prevent destruction of sulfhydryl groups. Aliquots of the hydroxylamine-treated reaction mixtures were assayed for activity after the indicated times.

appreciable restoration of activity was achieved within 20 to 90 minutes after addition of hydroxylamine. Figure 29 illustrates the reversal of inactivation and carbethoxy group removal as a function of time. Longer treatment with hydroxylamine resulted in reduction of activity even in control samples not treated with DEPC, so that no attempts were made to observe complete reactivation of DEPC-treated samples by longer incubation times.

Lineweaver Burk plots with either AMP, glucose-1-P, or glycogen as the variable substrate are shown in Figures 30, 31 and 32, and the calculated Michaelis parameters are shown in Table 11. The K_m values for both glucose-1-P and AMP increased with increasing reaction time and inactivation, both plots intersecting at a point to the left of the ordinate axis. The K_m value for glycogen remained unchanged after 2 minutes of reaction. After 5 minutes of reaction, the kinetics have become more complex, extrapolation of the plot including only the lower glycogen concentrations giving an unchanged K_m value, while extrapolation at the high glycogen concentrations gives a much larger K_m value. The latter result may suggest the existence of two enzyme species after 5 minutes of reaction. In general, the changes in both K_m and V_{max} values over the course of inactivation suggest the formation of catalytically active enzyme species with modified properties.

Ligand protection studies with phosphorylase b were inconclusive, as the allosteric activator AMP was found to retard the inactivation by itself reacting with DEPC. Glucose-1-P did not significantly alter the rate or extent of inactivation.

Figure 29.

Time course of reversal of DEPC reaction with phosphorylase b by hydroxylamine.

Phosphorylase b (0.5 mg/ml) was reacted with 2.3 mM DEPC at pH 6.0 for 5 minutes. The arrow at 5 minutes indicates the time of addition of hydroxylamine and DTT at pH 7.0 to final concentrations of 0.5M and 2.5 mM, respectively. The activity (X) and formation of carbethoxyhistidyl residues (O) were monitored as a function of time.

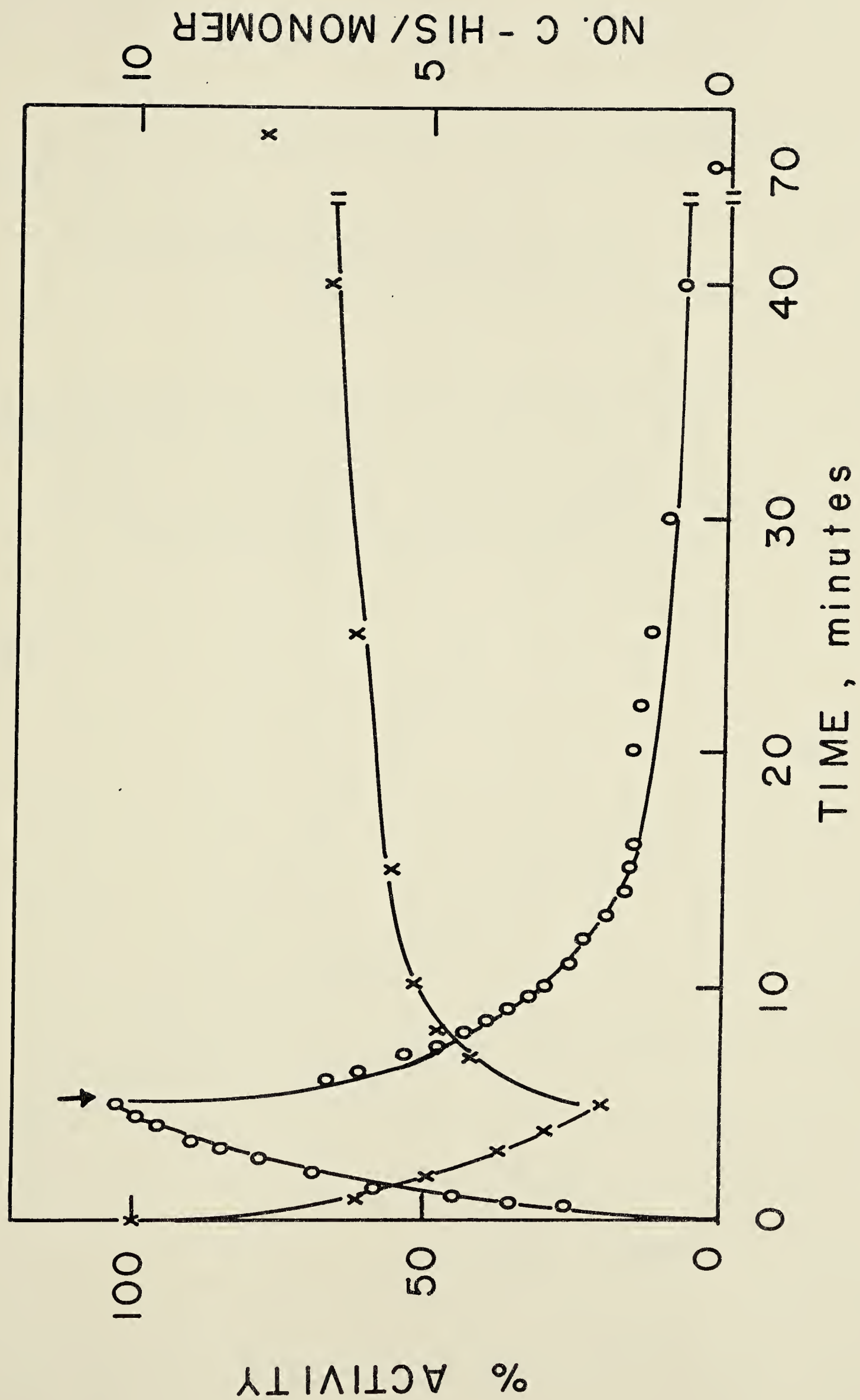
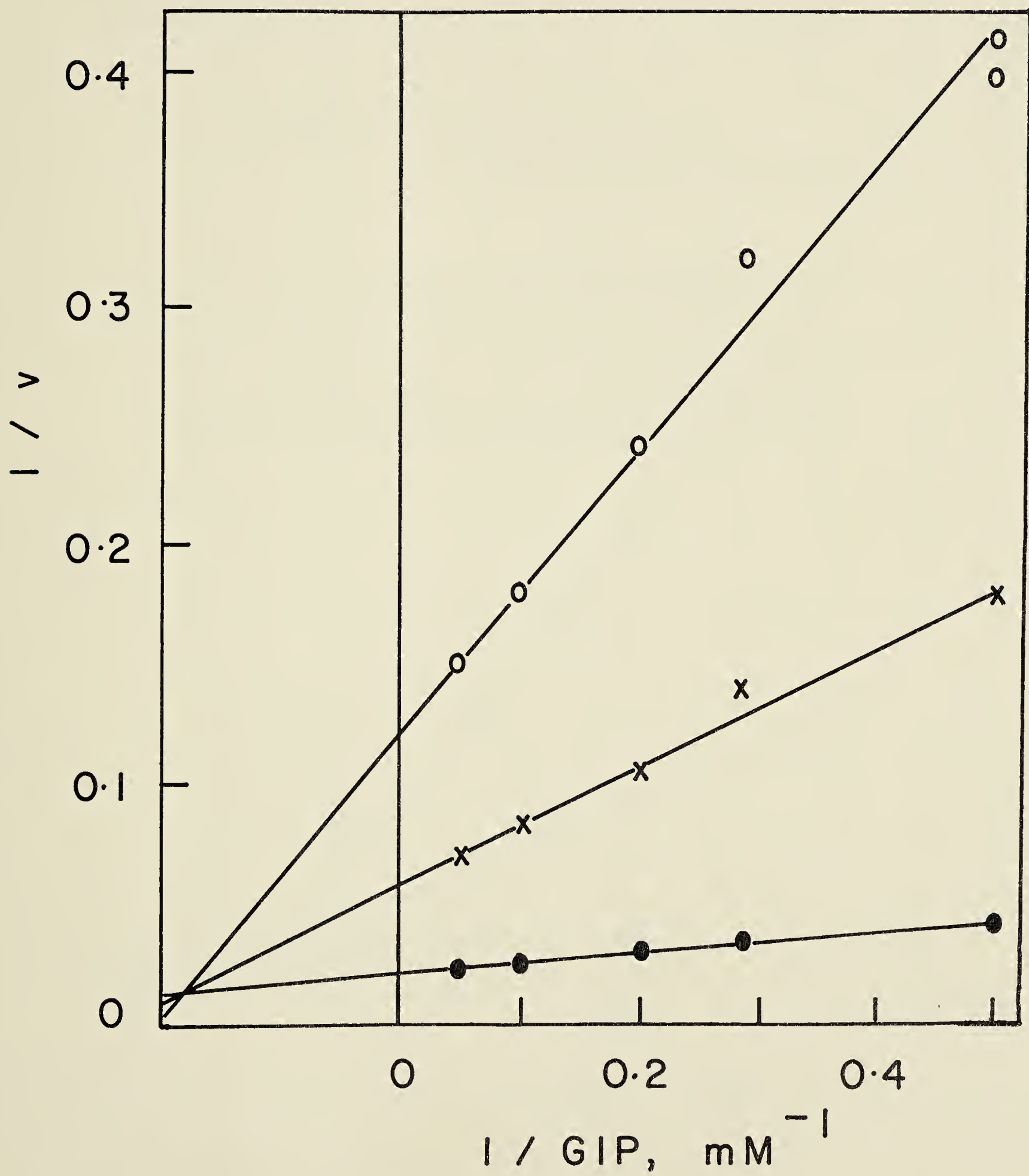




Figure 30.

Activity of DEPC-treated phosphorylase b as a function of glucose-1-P concentration.

0.5 mg/ml phosphorylase b was assayed for activity after 0 minutes (●), 2 minutes (X), and 5 minutes (O) of reaction with 2.3 mM DEPC at pH 6.15. Assays contained 1 mM AMP, 1% glycogen and varying glucose-1-P concentrations.



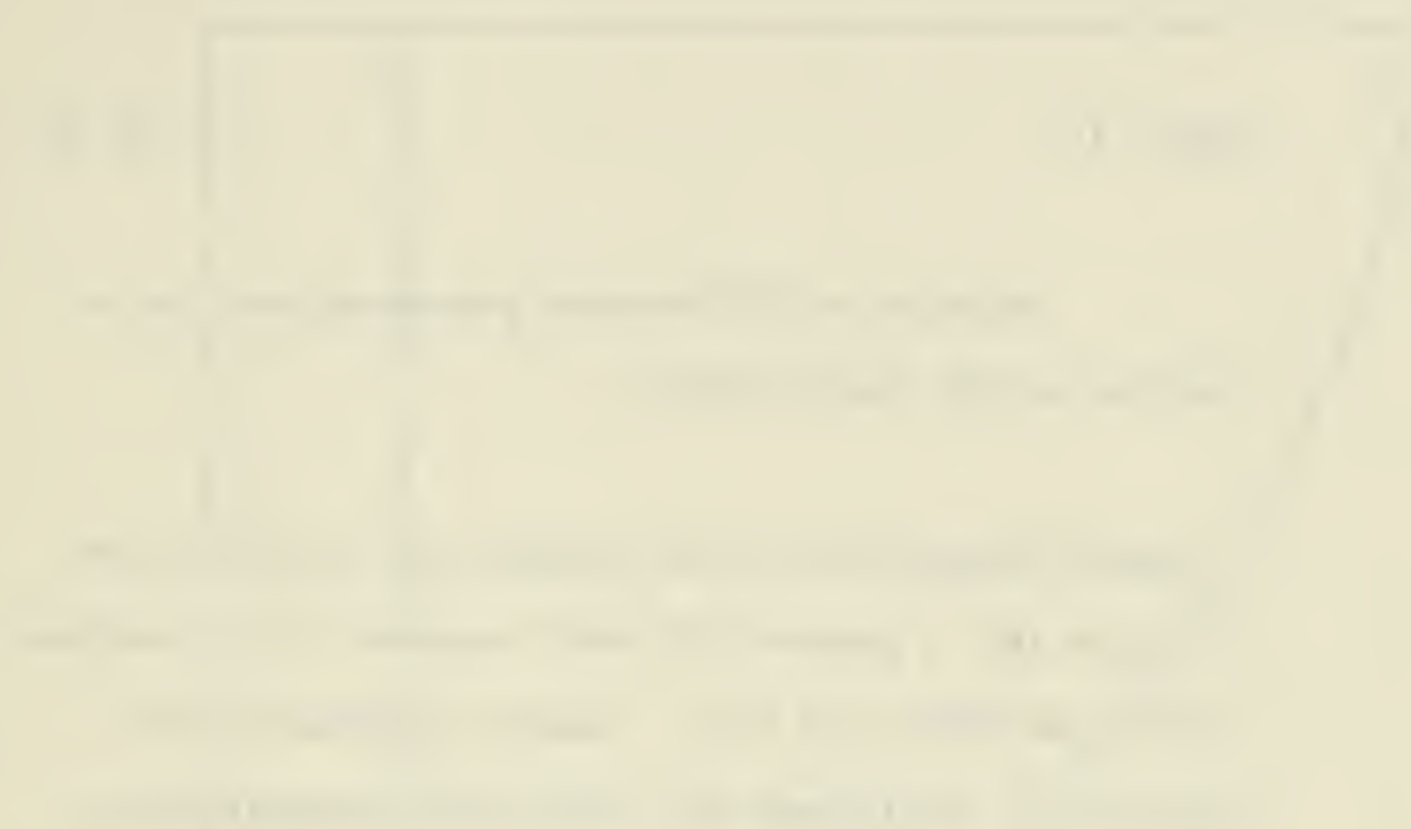


Figure 31.

Activity of DEPC-treated phosphorylase b as a function of AMP concentration.

0.5 mg/ml phosphorylase b was assayed for activity after 0 minutes (●), 2 minutes (X) and 5 minutes (○) of reaction with 2.3 mM DEPC at pH 6.15. Assays contained 24 mM glucose-1-P, 1% glycogen and varying AMP concentrations.

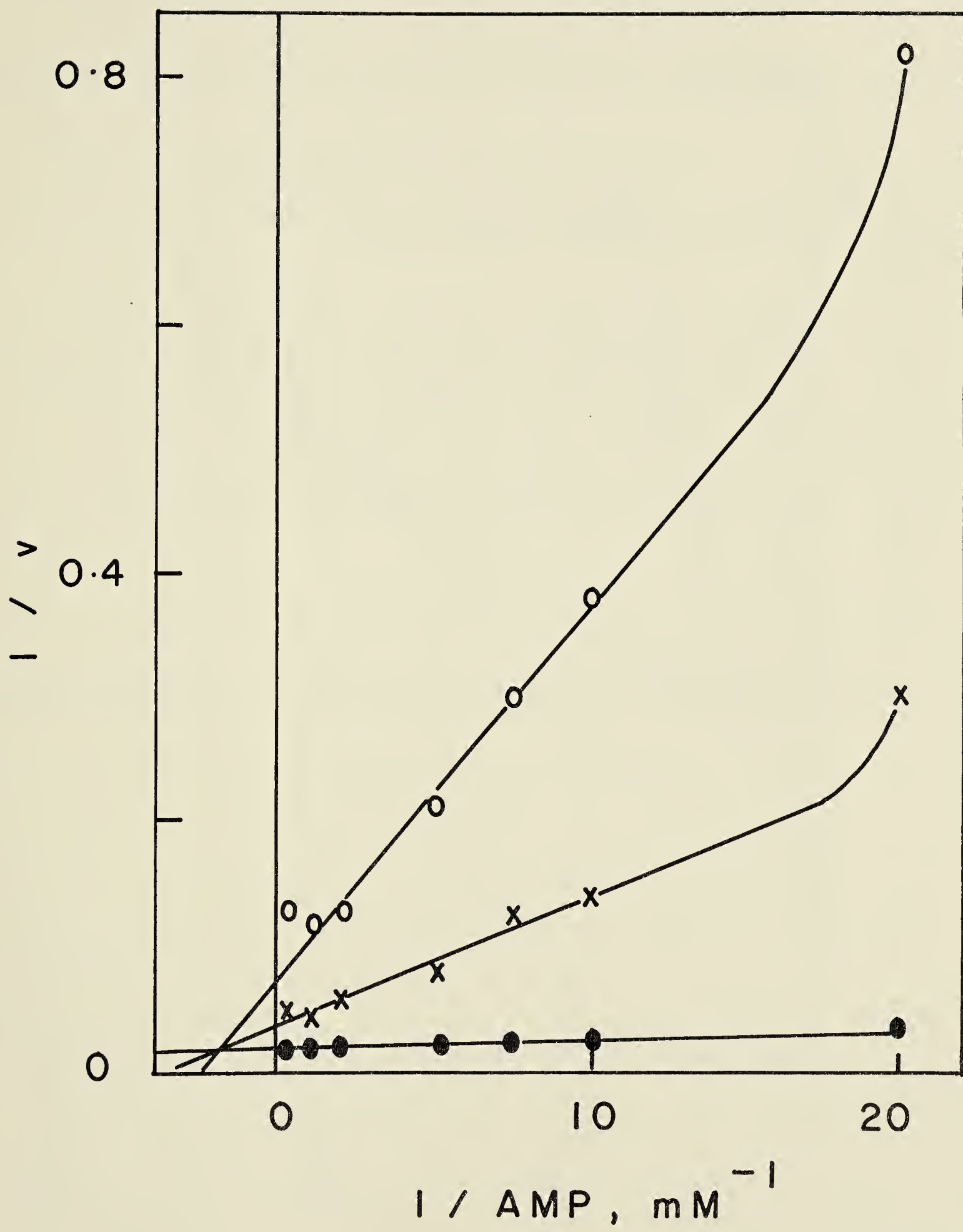




Figure 32.

Activity of DEPC-treated phosphorylase b as a function of glycogen concentration.

0.5 mg/ml phosphorylase b was assayed for activity after 0 minutes (●), 2 minutes (X) and 5 minutes (○) of reaction with 2.3 mM DEPC at pH 6.15. Assays contained 1 mM AMP, 24 mM glucose-1-P and varying glycogen concentrations.

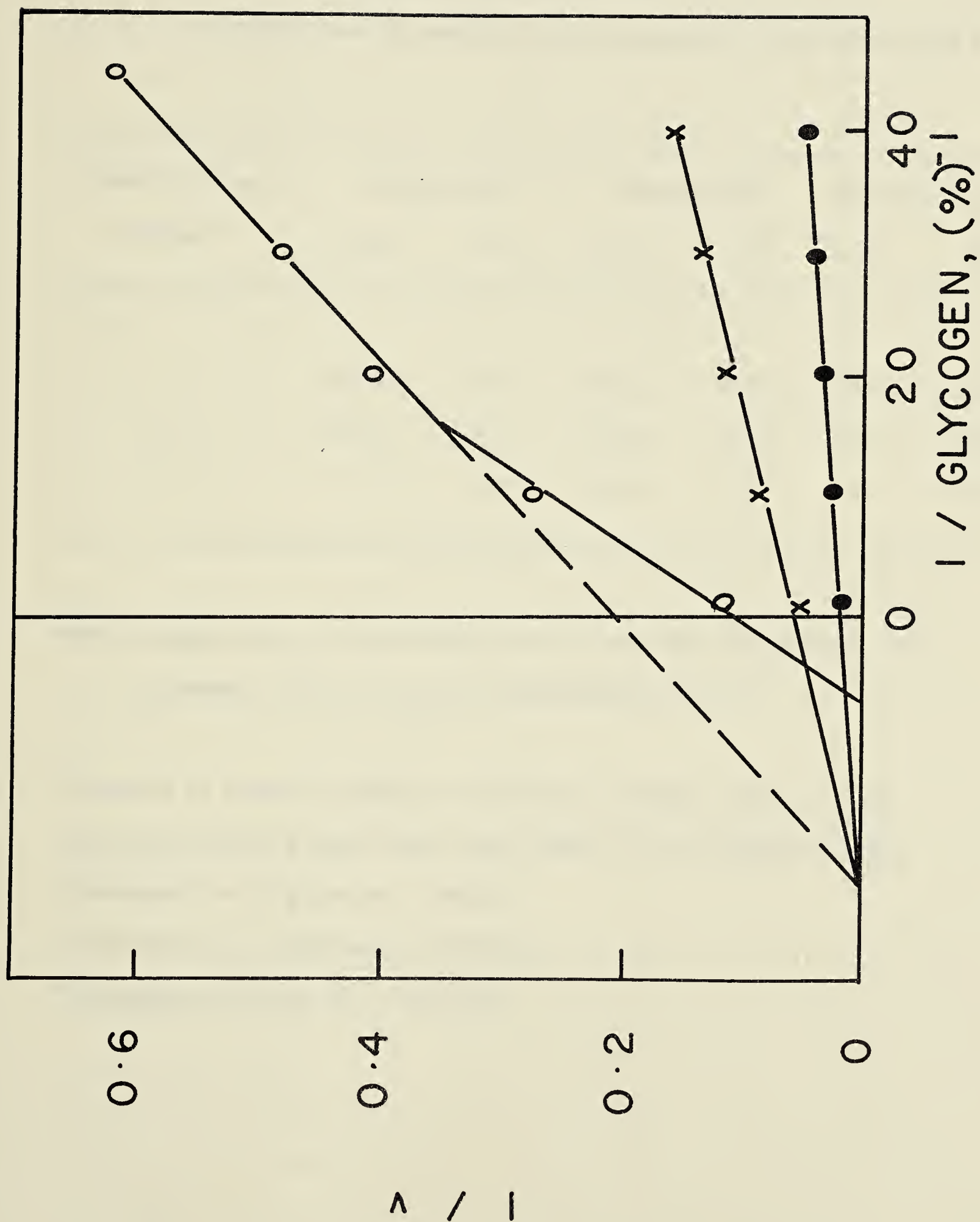


Table 11

Kinetic parameters for
phosphorylase b partially inactivated by 2.3 mM DEPC at pH 6

Reaction time, minutes ^a	Varying G1P ^b		Varying AMP ^c		Varying glycogen ^d	
	V _{max}	K _m	V _{max}	K _m	V _{max}	K _m
0	50.6	2.0	62.3	0.045	59.2	0.049
2	18.7	4.6	27.6	0.28	20.6	0.051
5	8.7	5.2	13.4	0.39	9.7	0.14

NOTE: Units for K_m are millimolar for G1P and AMP and percent for glycogen, and for V_{max} are μ moles/min/mg at 30°

^a aliquots of reaction mixture containing 0.5 mg/ml enzyme, 2.3 mM DEPC, pH 6, were diluted into assay buffer at the indicated times

^b determined at 1% glycogen, 1 mM AMP

^c determined at 1% glycogen, 24 mM G1P

^d determined at 24 mM G1P, 1 mM AMP

2. Phosphorylase a

Figure 33 illustrates the reaction of phosphorylase a with varying concentrations of DEPC at pH 6.0. It should be noted that only rough approximations of the concentrations of DEPC were reported in this experiment; accurate values are unknown since the purity of this batch of reagent had not been determined at the time (see Chapter II, section 5). However, these preliminary results indicate that the rates of inactivation were similar to those of phosphorylase b under similar conditions. The formation of carbethoxyhistidyl residues as a function of time is also illustrated in this figure, and shows that, like phosphorylase b, a great number of residues were modified also in phosphorylase a.

The inactivation of phosphorylase a by 0.35 mM DEPC at pH 6.8 was studied in the absence and presence of ligands. Incubation of the enzyme with either 25 mM glucose-1-P or 50 mM glucose or 50 mM glucose-6-P prior to addition of reagent did not significantly alter the rate or extent of inactivation.

C. Discussion

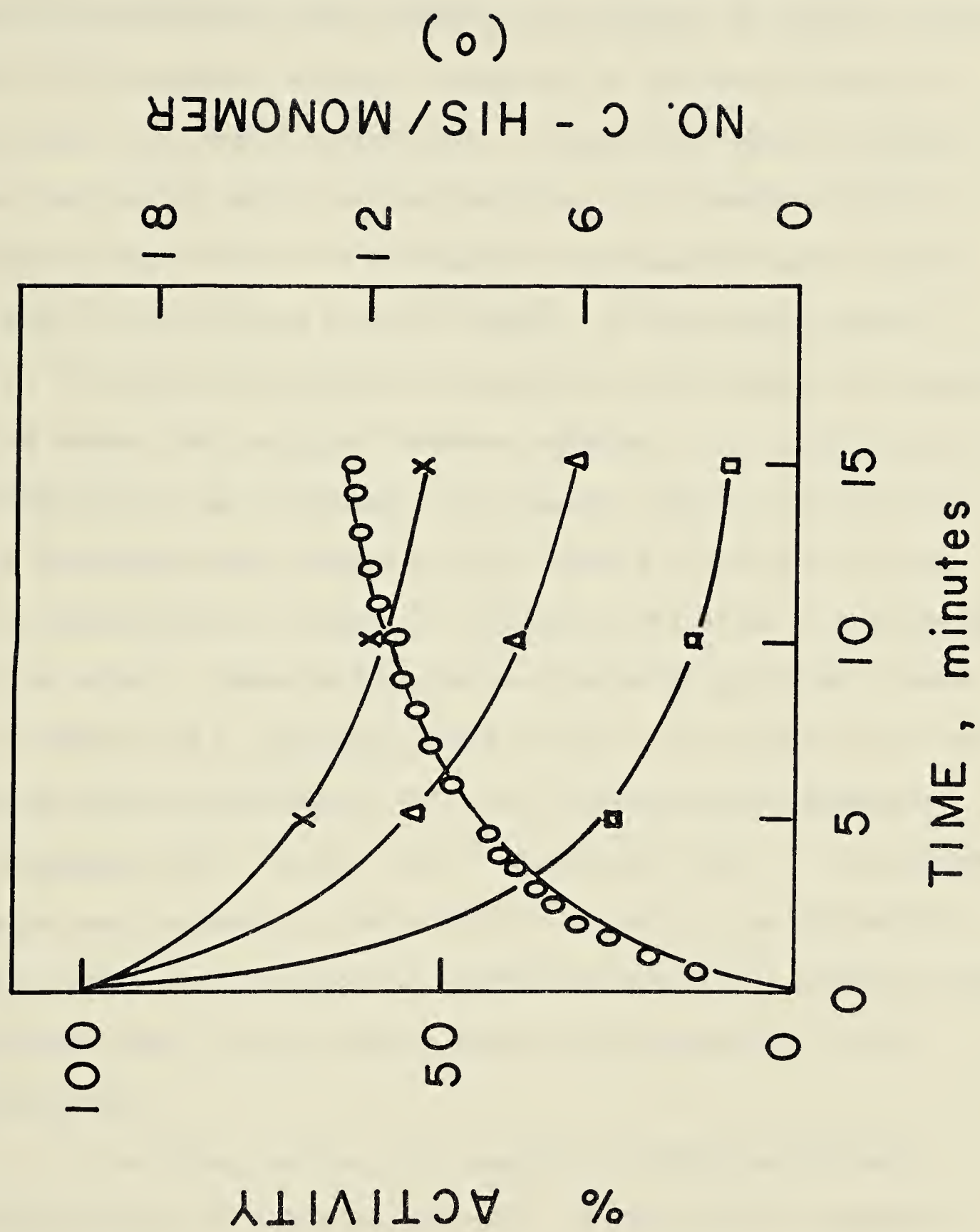
Both phosphorylases a and b are rapidly inactivated by DEPC in slightly acidic conditions. Several lines of evidence identify the inactivating reaction to be the modification of histidyl residues. These include ultraviolet spectral characteristics which are typical of carbethoxyhistidine formation, a pH dependence of inactivation in the range of pH values studied from 5.25 to 6.8, and the partial



Figure 33.

Time course of reaction of phosphorylase a with DEPC at pH 6.0.

Phosphorylase a (0.5 mg/ml) in 50 mM glycerophosphate-1 mM EDTA buffer at pH 6.0 was reacted with 0.46 mM (X), 0.92 mM (Δ) and 2.3 mM (\square) DEPC (see text). Aliquots of the reaction mixtures were assayed for activity at pH 6.8 at the indicated times. The formation of carbethoxyhistidyl residues (0) was calculated from $\Delta A_{240 \text{ nm}}$ for the reaction with 2.3 mM DEPC.



restoration of activity by hydroxylamine treatment at neutral pH.

However, while the selectivity of the reaction of DEPC for histidyl residues has been confirmed, the question of whether or not any of the modified residues is essential in substrate binding or catalytic roles remains unanswered. It seems that DEPC is a very reactive reagent capable of reacting with all accessible histidyl residues, and is unable to distinguish the essential ones, if any, in many of the proteins thus far studied. In our case, typically up to 15 residues per monomer were modified in the completely inactivated enzyme, and prolonged treatment resulted in virtually complete modification of all 19 residues per monomer. Numerous other workers have encountered this problem as well, which has led to difficulty in interpretation of "essential" residues in the midst of gross changes in the protein. These include the reaction with pig kidney D-amino acid oxidase and *C. adamanteus* venom L-amino acid oxidase (56), rabbit muscle lactate dehydrogenase (57), and glyceraldehyde-3-phosphate dehydrogenase (58). In the case of thermolysin (55), 13.4 ethoxyformyl groups were incorporated, but only 12.5 groups were incorporated in the reaction in the presence of competitive inhibitor, leading to the conclusion that a single group appears to be responsible for the inactivation.

In a few proteins, the reaction with DEPC selectively modifies only 1 or 2 histidyl residues. These include L-arginine oxygenase (56), octopine dehydrogenase (57), yeast alcohol dehydrogenase (59) and lactate dehydrogenase (26). No clear trend is apparent to explain the abnormal reactivity of these histidyl residues,

but hyperreactivity may be at least in part attributed to the tendency for DEPC to locate preferentially in very lipophilic regions, and to the limitation of DEPC nucleophilic reactivity to the nonprotonated base (52).

Clearly this work as well as the others on phosphorylase (9, 11) have indicated loss of activity concomitant with destruction of histidyl residues in phosphorylases a and b. However, the present study indicates that a great number of histidyl residues are modified, so that rather gross non-specific structural changes may be the cause of inactivation. The lack of any significant effect on inactivation by substrates and inhibitors tends to suggest that the site of inactivation is not likely to be localized at the active site or the anion-binding site. Without further evidence, it is not possible at this stage to confirm or deny the role of histidyl residues in the catalytic activity of phosphorylase.

Bibliography

1. Graves, D.J., & Wang, J.H. (1972). In "The Enzymes" (P.D. Boyer, ed.), 3rd edn, vol. 7., p.435, Academic Press, New York.
2. Zarkadas, C.G., Smillie, L.B. and Madsen, N.B. (1968). J. Mol. Biol. 38, 245.
3. Zarkadas, C.G., Smillie, L.B. and Madsen, N.B. (1970). Can. J. Biochem. 48, 763.
4. Battell, M.L., Smillie, L.B. and Madsen, N.B. (1968). Can. J. Biochem. 46, 609.
5. Battell, M.L., Zarkadas, C.G., Smillie, L.B. and Madsen, N.B. (1968). J. Biol. Chem. 243, 6202.
6. Huang, C.-C., and Madsen, N.B. (1970). Biochemistry 5, 116.
7. Wang, J.H. and Tu, J.-I. (1969). Biochemistry 8, 4403.
8. Kasvinsky, P.J. and Meyer, W.L. (1977). Arch. Biochem. Biophys. 181, 616.
9. Severson, D. and Madsen, N.B. Unpublished results.
10. Bailin, G. and Lukton, A. (1965). Biochim. Biophys. Acta 110, 622.
11. Kamogawa, A. and Fukui, T. (1975). Biochim. Biophys. Acta 403, 326.
12. Hulla, F.W. and Fasold, H. (1972). Biochemistry 11, 1056.
13. Anderson, R.A. and Graves, D.J. (1973). Biochemistry 12, 1895.
14. Anderson, R.A., Parrish, R.F. and Graves, D.J. (1973). Biochemistry 12, 1901.
15. Lee, Y.M. and Benisek, W.F. (1976). J. Biol. Chem. 251, 1553.
16. Fletterick, R.J., Sygusch, J. Semple, H. and Madsen, N.B. (1976). J. Biol. Chem. 251, 6142.

17. Kasvinsky, P.J. and Madsen, N.B. (1976). J. Biol. Chem. 251, 6852.
18. Fischer, E.H. and Krebs, E.C. (1962). Methods Enzymol. 5, 369.
19. Krebs, E.G., Love, D.S., Bratvold, J.E., Trayser, K.A., Meyer, W.L. and Fischer, E.H. (1964). Biochemistry 3, 1022.
20. Buc, M.H. and Buc, H. (1968). In "Symposium on regulation of enzyme activity and allosteric interactions", p. 109, Academic Press, New York.
21. Hedrick, J.L. and Fischer, E.H. (1965). Biochemistry 4, 1337.
22. Fletterick, R.J., Sygusch, J. Murray, N., Madsen, N.B. and Johnson, L.N. (1976). J. Mol. Biol. 103, 1.
23. Riordan, J.F. (1973). Biochemistry 12, 3915.
24. Thompson, G.W., Ockerman, L.T. and Schreyer, J.M. (1951). J. Am. Chem. Soc. 73, 1379.
25. Schreyer, J.M., Thompson, G.W. and Ockerman, L.T. (1950). Anal. Chem. 22, 1426.
26. Holbrook, J.J. and Ingram, V.A. (1973). Biochem J. 131, 729.
27. Daemen, F.J.M. and Riordan, J.F. (1974). Biochemistry 13, 2865.
28. Lobb, R.R., Stokes, A.M., Hill, H.A.O. and Riordan, J.F. (1975). FEBS Lett. 54, 70.
29. Marcus, F. (1975). Biochemistry 14, 3916.
30. Marcus, F. (1976). Biochemistry 15, 3505.
31. Powers, S.G. and Riordan, J.F. (1975). Proc. Nat. Acad. Sci. USA 72, 2616.
32. Riordan, J.F., McElvany, K.D. and Borders, C.L., Jr. (1977). Science 195, 884.
33. Takahashi, K. (1968). J. Biol. Chem. 243, 6171.

34. Means, G.E. and Feeney, R.E. (1971). In "Chemical modification of proteins", p. 195, Holden-Day Inc., San Francisco.
35. Marcus, F. Schuster, S.M. and Lardy, H.A. (1976). J. Biol. Chem. 251, 1775.
36. Sevilla, C.L. and Fischer, E.H. (1969). Biochemistry 8, 2161.
37. Kasvinsky, P.J. and Madsen, N.B. Manuscript in preparation.
38. Goff, H. and Murmann, R.K. (1971). J. Am. Chem. Soc. 93, 6058.
39. Audette, R.J., Quail, J.W., Black, W.H. and Robertson, B.E. (1973). Journal of Solid State Chemistry 8, 43.
40. Wells, A.F. (1950). In "Structural inorganic chemistry", 2nd edn., p. 343, Oxford Press, London.
41. Audette, R.J., Quail, J.W. and Smith, P.J. (1971). Tetrahedron Lett. 3, 279.
42. Shimomura, S. and Fukui, T. (1976). Biochemistry 15, 4438.
43. Kastenchmidt, L.L., Kastenchmidt, J. and Helmreich, E. (1968). Biochemistry 7, 3590.
44. Chao, J. and Graves, D.J. (1970). Biochem. Biophys. Res. Commun. 40, 1398.
45. Tu, J.-I., Jacobson, G.R. and Graves, D.J. (1971). Biochemistry 10, 1227.
46. Avramovic-Zikic, O., Breidenbach, W.C. and Madsen, N.B. (1974). Can. J. Biochem. 52, 146.
47. Breidenbach, W.C. (1972). In "Diazonium-1-H-tetrazole modification of phosphorylase", M. Sc. thesis, University of Alberta.
48. Hoare, D.G. and Koshland, D.E. (1967). J. Biol. Chem. 242, 2447.
49. Avramovic-Zikic, O., Shechosky, S. and Madsen, N.B. Personal communication.

50. Kasvinsky, P.J. Personal Communication.
51. Perfetti, R.B., Anderson, C.D., and Hall, P.L. (1976). *Biochemistry* 15, 1735.
52. Ehrenberg, L. Fedorcsak, I. and Solymosy, F. (1976). *Prog. Nucleic Acid Res. and Mol. Biol.* 16, 189.
53. Cousineau, J. and Meighen, E. (1976). *Biochemistry* 15, 4992.
54. Melchoir, W.B. and Fahrney, D. (1970). *Biochemistry* 9, 251.
55. Burstein, Y. Walsh, K.A. and Neurath, H. (1974). *Biochemistry* 13, 205.
56. Thomé-Beau, F., Lê-Thi-Lan, Olomucki, A. and Van Thoai, N. (1971). *Eur. J. Biochem.* 19, 270.
57. Huc, C., Olomucki, A., Lê-Thi-Lan, Pho, D.B. and Van Thoai, N. (1971). *Eur. J. Biochem* 21, 161.
58. Ovádi, J. and Keleti, T. (1969). *Acta Biochim et Biophys Acad Sci Hung* 4, 365.
59. Dickenson, C.J. and Dickinson, F.M. (1975). *Eur. J. Biochem.* 52, 595.
60. Titani, K., Koide, A., Hermann, J., Ericsson, L.H., Kumar, S., Wade, R., Walsh, K.A., Neurath, H. and Fischer, E. (1977). *Proc. Nat. Acad. Sci. USA*, in press.

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